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(71)
WISCONSIN ALUMNI RESEARCH FOUNDATION,
614 Walnut Street
13th Floor, MADISON, XX (US).

(72)
AHLQUIST, PAUL G. (US).
GERMAN, THOMAS L. (US).
RASOCHOVA, LADA (US).

(74)
SIM & MCBURNEY

(54) METHODES ET MATERIELS AMELIORES DE TRANSFORMATION
(54) IMPROVED METHODS AND MATERIALS FOR TRANSFORMATION

(57)
Disclosed herein are novel methods and materials directed to transforming a host cell and expressing exogenous RNA therein. Specifically disclosed are DNA-launching platforms used to introduce a replicating viral segment attached to an exogenous polynucleotide into a cell, whereby the exogenous polynucleotide is expressed in said cell and confers a detectable trait.

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(12) (19) (CA) **Demande-Application**

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(72) RASOCHOVA, LADA, US
(72) GERMAN, THOMAS L., US
(72) AHLQUIST, PAUL G., US
(71) WISCONSIN ALUMNI RESEARCH FOUNDATION, US
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(54) **METHODES ET MATERIELS AMELIORES DE TRANSFORMATION**

(54) **IMPROVED METHODS AND MATERIALS FOR TRANSFORMATION**

(57) L'invention concerne de nouvelles méthodes et de nouveaux matériels destinés à la transformation d'une cellule hôte et à l'expression dans celle-ci d'ARN exogène. Spécifiquement, l'invention concerne des plates-formes de lancement d'ADN utilisées pour introduire un segment viral réplicant fixé à un polynucléotide exogène dans une cellule, de manière que le polynucléotide exogène soit exprimé dans ladite cellule et confère un trait détectable.

(57) Disclosed herein are novel methods and materials directed to transforming a host cell and expressing exogenous RNA therein. Specifically disclosed are DNA-launching platforms used to introduce a replicating viral segment attached to an exogenous polynucleotide into a cell, whereby the exogenous polynucleotide is expressed in said cell and confers a detectable trait.

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(71) Applicant: WISCONSIN ALUMNI RESEARCH FOUNDATION [US/US]; 614 Walnut Street, Madison, WI 53705 (US).

(72) Inventors: RASOCHOVA, Lada; 5002 Sheboygan Avenue #326, Madison, WI 53705 (US). GERMAN, Thomas, L.; 1671 Sandy Rock Road, Hollandale, WI 53544 (US). AHLQUIST, Paul, G.; 3106 Bluff Street, Madison, WI 53705 (US).

(74) Agents: LLOYD, Jeff et al.; Saliwanchik, Lloyd & Saliwanchik, P.A., Suite A-1, 2421 N.W. 41st Street, Gainesville, FL 32606-6669 (US).

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(57) Abstract

Disclosed herein are novel methods and materials directed to transforming a host cell and expressing exogenous RNA therein. Specifically disclosed are DNA-launching platforms used to introduce a replicating viral segment attached to an exogenous polynucleotide into a cell, whereby the exogenous polynucleotide is expressed in said cell and confers a detectable trait.

DESCRIPTION

IMPROVED METHODS AND MATERIALS FOR TRANSFORMATION

This invention was made with United States government support awarded by the following agency:

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The United States has certain rights in this invention.

Background of the Invention

RNA viruses have been found to be valuable tools in the phenotypic and genotypic transformation of targeted cells and tissues. See, *e.g.*, U.S. Patent No. 5,500,360, which teaches novel viral RNA expression vectors. It has been shown that the RNA of the genome of an RNA virus can be modified to include an exogenous RNA segment and that the modified RNA can be introduced into a host cell, replicated therein, and thereby express the exogenous RNA segment.

Current methods of inoculating a host cell with modified RNA viruses involve the *in vitro* transcription of a particular strand followed by the introduction of the resulting RNA transcripts into the host cell. One problem with the current inoculation method is that the RNA rapidly degrades which causes a low efficiency of infection. In addition, the preparation of the *in vitro* RNA transcripts is expensive and time consuming.

Further, with the advent of transformation and the genetic engineering of plants, much concern has arisen concerning the potential hazard of the dispersal of dangerous traits into the environment. For example, genes increasing the stress tolerance and/or herbicide resistance of an agriculturally important crop could theoretically "leak" to surrounding less desirable and damaging plants, *e.g.*, through pollen, mechanical or insect dispersal. This phenomenon could create a novel species of "super-weed" which could wreak havoc on the agricultural industry. Existing RNA virus-based vectors can spread to non-target plants by mechanical means and/or by insects. Such spread can be prevented by using vectors that can replicate and/or move only in target plants expressing the appropriate trans-acting factors. Accordingly, there remains a need for less expensive and more efficient methods of transformation of target cells and tissues. Moreover, there is a need for a novel method of transformation which alleviates the potential dangers associated with the unwanted spread of engineered traits into the environment.

Brief Summary of the Invention

The subject invention pertains to improved materials and methods for transforming host cells which involve transfecting said cells with a DNA-launching platform. One aspect of the subject invention pertains to a DNA-launching platform which encodes a modified viral RNA molecule downstream of DNA-dependent RNA polymerase (pol) promoter, whereby the DNA-launching platform is capable of being introduced into a host cell and effectively "launching" said modified viral RNA molecule into the host cell such that it is replicated and expressed therein. The term "modified viral RNA molecule" as used herein refers to a viral RNA which has been changed from its natural state. Examples of changes of viral RNA include, but are not limited to, removal of a part of viral RNA genome, insertion or substitution of an exogenous RNA, etc. The exogenous RNA segment can be located in a region of the viral RNA molecule such that it does not disrupt the RNA replication. Techniques for such manipulations have been well known to those of ordinary skill in the art for many years. Preferably, the modified viral RNA molecule further comprises a ribozyme which is located in the proximity of the 3' end of the modified viral RNA molecule. The viral segment may have the ability to be replicated with or, alternatively, without the presence of trans-acting viral replicating elements.

Another aspect of the subject invention pertains to a method of genotypically or phenotypically modifying a host cell, comprising introducing a DNA-launching platform which encodes a viral RNA molecule and an exogenous RNA segment in a location which does not disrupt the replication of said viral RNA segment or said exogenous RNA segment, whereby the exogenous RNA segment confers a detectable trait in the host cell. The subject invention applies to a wide array of plant cells.

Still a further aspect of the subject invention pertains to cells in which the DNA-launching platform of the subject invention has been introduced.

Yet another aspect of the subject invention pertains to a plant comprising cells transfected with the DNA-launching platform.

The novel methods and materials of the subject invention provide a greater inoculation efficiency of RNA viruses because use of DNA-launching platforms of the subject invention are more resistant to degradation than RNA inocula, and because each DNA platform produces multiple RNA transcripts over an extended period of time. As the DNA-launching platform provides a genetically stable *in planta* archive copy of a desired vector construct, the continuing transcription of said DNA platform will repeatedly reinoculate the host cell with the desired construct. This serves to counteract genetic instability problems that have inhibited the expression of some genes from vectors based on plant and animal RNA viruses. Further, the

inoculation methods of the subject invention provide a much simpler means of producing inocula in bulk for large scale use, which is cheaper and more efficient than inoculating with *in vitro* RNA transcripts.

Brief Description of the Drawings

Figure 1 represents the schematic for producing the 1a and 2a proteins in the host cell.

Figure 2 illustrates an example of an *Agrobacterium* transformation vector containing an expression cassette capable of expressing 1a and/or 2a BMV proteins.

Figure 3 illustrates several *Agrobacterium* vectors that were produced to transform host plant cells (black rectangles indicate T-DNA borders).

Figure 4 represents the general mechanism of BMV RNA3 launching, and replication.

Figure 5 depicts DNA-launching platforms which can be used in accord with the teachings contained herein. The BMV and CCMV designations denote cis-acting elements.

Figure 6 depicts DNA-launching platforms which can be used in accord with the teachings contained herein.

Figure 7 depicts DNA-launching platforms which can be used in accord with the teachings contained herein.

Figure 8 depicts DNA-launching platforms which can be used in accord with the teachings contained herein.

Figure 9 depicts *Agrobacterium* vector for delivery of DNA-launching platforms to plant cells (open triangles represent T-DNA borders).

Figure 10 depicts DNA-launching platforms which can be used in accord with the teachings contained herein.

Legend For Figures 5-10:

35S	=	CaMV35S promoter
t	=	termination/polyA + sequences
Rz	=	ribozyme
NOS	=	NOS promoter
OOA	=	origin of assembly
FG	=	foreign gene

Figure 11 shows that BMV replication factors support efficient RNA3 replication in protoplasts.

Figure 12 shows the efficient replication of launched BMV RNA3 in protoplasts.

Figure 13 shows transgenic expression of BMV 1a and 2a mRNAs in *N. tabacum* and *N. benthamiana*.

Figure 14 shows the efficient replication of launched BMV RNA3 in (1a + 2a)-transgenic plants.

Figure 15 shows the successful GUS expression from the launched BMV RNA3 in (1a + 2a)- transgenic plants.

Figure 16 shows the successful GUS expression from the launched BMV RNA3 in protoplasts.

Figure 17 shows the successful GFP expression from the launched BMV RNA3 in (1a + 2a) - transgenic plants.

Figure 18 shows the successful GFP expression from the launched BMV RNA3 in protoplasts.

Figure 19 shows the efficient replication of the launched BMV RNA3 in (1a + 2a)-transgenic *N. benthamiana* using *Agrobacterium* inoculation.

Figure 20 shows the successful GUS expression from the launched BMV RNA3 having the SHMV coat protein in (1a + 2a)-transgenic plants.

Figure 21 shows that launched BMV replicates, moves cell-to-cell, and spreads long distances in (1a+2a)-transgenic plants.

Figure 22 shows transfection of progeny from (1a+2a)-transgenic *N. benthamiana* with BMV RNA3 DNA-launching platform and localization of the launched RNA3 to the roots.

Brief Description of the Sequences

SEQ ID NO. 1: pB1LR2 – partial nucleotide sequence includes BMV 1a expression cassette.

SEQ ID NO. 2: pB1LR3 – partial nucleotide sequence includes BMV 1a expression cassette.

SEQ ID NO. 3: pB2LR4 – partial nucleotide sequence includes BMV 2a expression cassette.

SEQ ID NO. 4: pB2LR5 – partial nucleotide sequence includes BMV 2a expression cassette.

SEQ ID NO. 5: pB12LR6 – partial nucleotide sequence includes BMV 1a and 2a expression cassettes.

SEQ ID NO. 6: pB12LR7 – partial nucleotide sequence includes BMV 1a and 2a expression cassettes.

SEQ ID NO. 7: pB12LR8 – partial nucleotide sequence includes BMV 1a and 2a expression cassettes.

SEQ ID NO. 8: pB12LR9 – partial nucleotide sequence includes BMV 1a and 2a expression cassettes.

Detailed Disclosure of the Invention

To facilitate understanding of the invention, certain terms used throughout are herein defined. The term "RNA virus" as used herein means a virus whose genome is RNA in a double-stranded or single-stranded form, the single strand being a (+) strand or (–) strand.

The terms "transfection" or "transfected" as used herein means an introduction of a foreign DNA or RNA into a cell by mechanical inoculation, electroporation, agroinfection, particle bombardment, microinjection, or by other known methods.

The terms "transformation" or "transformed" as used herein means a stable incorporation of a foreign DNA or RNA into the cell which results in a permanent, heritable alteration in the cell. Accordingly, the skilled artisan would understand that transfection of a cell may result in the transformation of that cell.

The term "launched" as used herein refers to a polynucleotide that has been transcribed from a DNA-launching platform, as described herein and, preferably, replicated.

The term "cis-acting element" as used herein denotes that portion of the RNA genome of an RNA virus which must be present in cis, that is, present as a part of each viral strand as a necessary condition for replication of that strand. Virus replication may depend upon the existence of one or more trans (diffusible) elements which interact with the cis-acting element to carry out RNA replication. If trans-acting elements are necessary for replication, they need not be present or coded for on the modified viral RNA provided, but may be made available within the infected cell by some other means. For example, the trans-acting replication functions may be provided by other, unmodified or modified, components of the viral genome transfected into the cells simultaneously with the modified RNA. The same approach can be used for other trans-acting functions including movement protein, coat protein, and other functions. The target cell may also be premodified, for example, cells may have been previously transformed to provide constitutive expression of the trans-acting functions from a chromosome. The cis-acting element is composed of one or more segments of viral RNA which must be present on any RNA molecule that is to be replicated within a host cell by RNA replication. The segment will most

likely be the 5′ and 3′ terminal portions of the viral RNA molecule, and may include other portions and/or virus open reading frames as well. The cis-acting element is accordingly defined in functional terms: any modification which destroys the ability of the RNA to replicate in a cell known to contain the requisite trans-acting elements, is deemed to be a modification in the cis-acting element. Conversely, any modification, such as deletion or insertion in a sequence region which is able to tolerate such deletion or insertion without disrupting replication, is a modification outside the cis-acting element. As is demonstrated herein, using the example of BMV which is known and accepted by those skilled in the art to be a functional example from which substantial portions of an RNA virus molecule may be modified, by deletion, insertion, or by a combination of deletion and insertion, without disrupting replication.

"Exogenous RNA" is a term used to describe a segment or component of RNA to be inserted into the virus RNA to be modified, the source of the exogenous RNA segment being different from the RNA virus itself. The source may be another virus, an organism such as a plant, animal, bacteria, virus, or fungus. The exogenous RNA may be a chemically synthesized RNA, derived from a native RNA, or it may be a combination of the foregoing. The exogenous RNA may provide any function which is appropriate and known to be provided by an RNA segment. Such functions include, but are not limited to, a coding function in which the RNA acts as a messenger RNA encoding a sequence which, when translated by the host cell, results in synthesis of a peptide or protein having useful or desired properties; the RNA segment may also be structural, as for example in ribosomal RNA; it may be regulatory, as for example with small nuclear RNAs or anti-sense RNA; or it may be catalytic. One skilled in the art will understand that the exogenous RNA may encode, for example, a protein which is a key enzyme in a biochemical pathway, which upon expression effects a desirable phenotypic characteristic, such as altering cell metabolism. Further, the exogenous RNA may encode a protein involved in transcriptional regulation, such as zinc finger, winged-helix, and leucine-zipper proteins. A particularly interesting function is provided by anti-sense RNA, sometimes termed (−) strand RNA, which is in fact a sequence complementary to another RNA sequence present in the target cell which can, through complementary base pairing, bind to and inhibit the function of the RNA in the target cell.

The term "non-viral" is used herein in a special sense to include any RNA segment which is not normally contained within the virus whose modification is exploited for replication and expression, and is therefore used synonymously with "exogenous". Accordingly, a gene derived from a different virus species than that which is modified is included within the meaning of the terms "non-viral" and "exogenous" for the purposes of describing the invention. For

example, a non-viral gene as the term is used herein could include a gene derived from a bacterial virus, an animal virus, or a plant virus of a type distinguishable from the virus modified to effect transformation. In addition, a non-viral gene may be a structural gene derived from any prokaryotic or eukaryotic organism.

In one embodiment, the subject invention concerns a novel method of transfecting a host cell which uses a DNA-launching platform to introduce viral RNA into the cell. The subject invention is directed towards a method of transfection employing a DNA-launching platform which encodes a modified viral RNA molecule comprising an RNA viral component attached to an exogenous RNA component and a DNA-dependent RNA pol promoter. The DNA-dependent RNA pol promoter is preferably but not necessarily fused within up to 10 nucleotides of the 5′ transcriptional start site of the modified viral RNA molecule, and more preferably within up to 5 nucleotides of the 5′ transcriptional start site. Expression of the DNA-launching platform produces transcripts of the modified viral RNA molecule that are then capable of RNA replication in the presence of replication factors, which can be present in the modified viral RNA and/or may be supplied in trans by other means including expression from chromosome or supplied on different launching plasmids. When the modified viral RNA is replicated, the exogenous RNA can be replicated as well. Further, the exogenous RNA can be expressed in the cell, thereby providing a predetermined phenotypic characteristic. In a preferred embodiment, the DNA launching platform further comprises a nucleotide sequence encoding a self-cleavable ribozyme situated proximate to the 3′ end of said RNA molecule. As would be readily apparent to those skilled in the art, known ribozymes may be used in accordance with the subject invention. In a preferred embodiment, the ribozyme cleaves the modified RNA viral molecule at the 3′ region. The 3′ region can consist of up to 30 nucleotides upstream or downstream of the 3′ end; and preferably consists of up to 10 nucleotides upstream or downstream of the 3′ end. In a more preferred embodiment, the ribozyme cleaves the modified RNA viral molecule precisely at the 3′ end. Other known regulatory sequences, *e.g.*, promoters and/or termination sequences, may also be substituted for and/or included on the DNA-launching platform. A suitable restriction site can be introduced proximate to the 3′ end of the modified viral RNA molecule sequence and the DNA molecule can be cleaved by an appropriate restriction enzyme prior to transfection. The term "DNA-launching platform" as used herein is intended to mean a DNA molecule, circular or linear, which has a coding region comprising a segment encoding a modified viral RNA segment, and further, which is capable of being delivered into a cell and subsequently transcribed.

Possible regulatory sequences can include, but are not limited to, any promoter already shown to be constitutive for expression, such as those of viral origin (CaMV 19S and 35S) or so-called "housekeeping" genes (ubiquitin, actin, tubulin) with their corresponding termination/polyA + sequences. Also, seed-and/or developmentally-specific promoters, such as those from plant fatty acid/lipid biosynthesis genes (ACPs, acyltransferases, desaturases, lipid transfer protein genes) or from storage protein genes (zein, napin, cruciferin, conglycinin, phaseolin, or lectin genes, for example), with their corresponding termination/polyA + sequences can be used for targeted expression. In addition, the gene can be placed under the regulation of inducible promoters and their termination sequences so that gene expression is induced by light (rbcS-3A, cab-1), heat (hsp gene promoters) or wounding (mannopine, HGPGs). It is clear to one skilled in the art that a promoter may be used either in native or truncated form, and may be paired with its own or a heterologous termination/polyA + sequence.

In a particularly preferred embodiment, the subject invention is directed toward a method of genotypically or phenotypically modifying a cell comprising the following steps: a) forming a cDNA molecule of a virus RNA, or of at least one RNA component if the RNA virus is multipartite, the viral RNA having been modified to contain a DNA segment encoding a non-viral RNA component situated in a region able to tolerate such insertion without disrupting replication of the RNA product encoded thereby; b) cloning modified cDNA into a DNA-launching platform; and c) transfecting a suitable host cell with said DNA-launching platform. In a most preferred embodiment, the method further comprises pretransforming a plant with trans-acting viral replication factors and/or other trans-acting factors. Such trans-acting factors may include viral movement proteins(s), coat protein(s), viral protease(s), and other structural and nonstructural genes. In addition to stable expression of trans-acting factors, trans-acting factors may be introduced on separate expression plasmids or may be expressed from RNA transcripts. In a preferred embodiment such trans-acting factors do not replicate. Suitable host cells may include protoplasts, cells in suspension, or cells in tissues or whole organisms.

In a specific embodiment intended as an example of the broader teachings herein, the RNA viral segment can be derived from brome mosaic virus (BMV), whereby the DNA-launching platform comprises DNA encoding the RNA3 segment of the virus. Brome mosaic virus (BMV) is a member of the α virus-like super family of positive-strand RNA viruses of animals and plants, and has a genome divided among three RNAs. RNA1 and RNA2 encode the 1a and 2a proteins, respectively, which are necessary for a genomic RNA replication and subgenomic mRNA synthesis (see, *e.g.*, U.S. Patent No. 5,500,360, which to the extent not inconsistent herewith, is incorporated herein by reference). These proteins contain three

domains conserved in all other members of the α virus-like super family. 1a (109kDa) contains a c-proximal helicase-like domain and an n-proximal domain implicated in RNA capping, and 2a (94kDa) contains a central polymerase-like domain. See, *e.g.*, French and Ahlquist, (1988). 1a and 2a interact with each other and with cell factors to form a membrane bound viral RNA replication complex associated with the endoplasmic reticulums of infected cells. BMV RNA3, a 2.1-kb RNA, encodes the 3a protein (32kDa) and coat protein (20kDa), which are involved in the spread of BMV infection in its natural plant hosts but are dispensable for RNA replication. See U.S. Patent No. 5,500,360. The 3a or coat protein gene of the RNA3 viral segment can be replaced with exogenous RNA, whereby it does not interfere with the replication element. Further, the exogenous RNA segment can be inserted downstream of an additional subgenomic promoter. Still further, cells or tissues can be pretransformed to express 1a, 2a, 3a, and coat protein, or any combination thereof, wherein DNA-launching platforms containing a foreign gene(s) with the necessary cis-acting components is transfected, such that the foreign gene is replicated and/or expressed.

In one embodiment, the host cell is pretransformed with BMV1 or BMV2 such that it is transgenically engineered to express 1a and 2a proteins. Preferably, the 5′ and 3′ ends of BMV1 and BMV2 are removed such that they are incapable of replication, but can express 1a and 2a to form a viral RNA replication complex associated with the endoplasmic reticulum of the host cell. Subsequent transfection of a DNA-launching platform comprising the RNA3 viral replication segment, as well as the exogenous RNA of interest, can produce the expression of said exogenous RNA while also preventing the undesired and dangerous spread of viral RNA spillage into the environment. That is, because a plant must have all 3 segments to form infectious BMV particle(s), problems associated with the environmentally hazardous escape of foreign genes through mechanical or insect dispersal of RNA virus vectors are avoided. One skilled in the art will readily appreciate that in the example of BMV that DNA-launching platforms could be also derived from either RNA1 or RNA2. For example, the sequence encoding the 1a protein could be replaced with an exogenous RNA; replication would require the expression of 1a (*e.g.*, separate expression plasmid). In a preferred embodiment, the DNA-launching platform also comprises a ribozyme situated proximate to the 3′ end of the modified RNA3, wherein said ribozyme cleaves the RNA3 at the 3′ end. As would be readily apparent to the skilled artisan with the teachings contained herein, viral segments from other known viruses, and/or subviral agents, can be used to formulate DNA-launching platforms of the subject invention. One skilled in the art will appreciate that BMV is merely one representative example of the many viruses suitable for practicing the subject invention. It is widely accepted that

principles on which the subject invention is based are broadly applicable to a myriad of viruses. Examples of other such viruses include, but are not limited to, alfalfa mosaic virus (AMV), barley stripe mosaic virus, cowpea mosaic virus, cucumber mosaic virus, reoviruses, polio virus, sindbis virus, vesicular stomatitis virus, influenza virus, retroviruses, and cowpea chlorotic mottle virus (CCMV) and any other viruses that replicate through RNA intermediates and from which a cDNA copy can be obtained. Specifically, as the other viruses are further characterized, those of skill in the art will readily appreciate the applicability of the teachings herein to other suitable viruses as well.

The skilled artisan would easily appreciate that known methods of introducing foreign DNA into cells can be used in accordance with the teachings of the subject disclosure. Such methods include, but are not limited to, mechanical inoculation, particle bombardment, agroinfection, electroporation, and microinjection, as well as other known methods.

Various aspects of the invention can be modified as needed, depending upon specific characteristics of the virus selected as the transforming and transfecting agent and of the RNA segment to be inserted. For example, the inserted gene need not be a naturally occurring gene, but may be modified, a composite of more than one coding segment, or it may encode more than one protein. The RNA may also be modified by combining insertions and deletions in order to control the total length or other properties of the modified RNA molecule. The inserted non-viral gene may be either prokaryotic or eukaryotic in origin. The inserted gene may contain its own translation start signals, for example, a ribosomal binding site and start (AUG) codon, or it may be inserted in a manner which takes advantage of one or more of these components preexisting in the viral RNA to be modified. Certain structural constraints must be observed to preserve correct translation of the inserted sequence, according to principles well understood in the art. For example, if it is intended that the exogenous coding segment is to be combined with an endogenous coding segment, the coding sequence to be inserted must be inserted in reading frame phase therewith and in the same translational direction.

It will be understood by those ordinarily skilled in the art that there may exist certain genes whose transfer does not result in obvious phenotypic modification of the recipient cell. Such may occur, for example, if the translation product of the non-viral gene is toxic to the host cell, is degraded or processed in a manner which renders it non-functional or possesses structural features which render it impossible for the host cell to translate in sufficient quantities to confer a detectable phenotype on the transformed cells. However, the invention does not depend upon any specific property of an RNA segment or gene being transferred. Therefore, the possible existence of RNA segments or genes which fail to confer a readily observable phenotypic trait

on recipient cells or plants is irrelevant to the invention, and in any case will be readily recognizable by those of ordinary skill in the art without undue experimentation.

An exogenous RNA segment may be inserted at any convenient insertion site in any of the cDNA sequences corresponding to a viral RNA, or component RNA of a multipartite RNA virus, provided the insertion does not disrupt a sequence essential for replication of the RNA within the host cell. For example, for a virus whose coat protein is not essential for replication, an exogenous RNA segment may be inserted within or substituted for the region which normally codes for coat protein. As desired, regions which contribute to undesirable host cell responses may be deleted or inactivated, provided such changes do not adversely affect the ability of the RNA to be replicated in the host cell. For many single component and multipartite RNA viruses, a reduction in the rate of normal RNA replication is tolerable and will in some instances be preferred, since the amount of RNA produced in a normal infection is more than enough to saturate the ribosomes of the transformed cell.

Plant cells which are inoculated in culture will normally remain transfected as the cells grow and divide since the RNA components expressed from the DNA-launching platform are able to replicate and thus become distributed to descendant cells upon cell division. Plants regenerated from phenotypically modified cells, tissues, or protoplasts remain phenotypically modified. Similarly, plants transfected as seedlings remain transfected during growth. Optimal timing of application of the transfecting components will be governed by the result which is intended and by variations in susceptibility to the transfecting components during various stages of plant growth.

Many plant RNA viruses are seed transmitted from one generation to the next. This property can be exploited to effect genotypic transformation of a plant. That is to say, the modified RNA remains transmissible from one generation to the next, just as seed-borne virus infections are transmitted from one generation to the next.

Following are examples which illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 – Construction of *Agrobacterium* Vectors

Binary vectors for expressing the BMV 1a and 2a proteins in plants were constructed. Starting with the pBI101.2 construct (Clontech, Palo Alto, CA), the GUS gene was removed by first cutting the construct with EcoRI and SnaBI. The overhanging restriction fragment ends

were filled in by treatment with Klenow fragments and dNTPs. The restriction fragment ends were religated forming the pB101.2LR1.

The 2a expression cassette was inserted into pBI101.2 LR1. First the pBI101.2LR1 was cut with Hind III and dephosphorylated. Next, pB2PA17 (Dinant *et al.*, 1993) was cut with Hind III and the 2a insert was purified using a low melting agarose gel. The restriction fragment ends were ligated forming the pB2LR4 and pB2LR5 (Figures 3c and 3d).

The 1a expression cassette was inserted into pBI101.2LR1 by first cutting pBI101.2LR1 with SnaBI and dephosphorylated. pB1PA17 (Dinant *et al.*, 1993) was cut with PstI and the extra nucleotides were removed with T4 DNA polymerase. The 1a insert was purified using a low melting agarose gel. The restriction fragment ends were ligated forming the pB1LR2 and pB1LR3 vectors (Figures 3a and 3b).

The 1a expression cassette was inserted into pB2LR4 and pB2LR5 by cutting pB2LR4 or pB2LR5 with SnaBI and dephosphorylated. PB1PA17 (Dinant *et al.*, 1993) was cut with PstI, and the extra nucleotides were removed with T4 DNA polymerase. The 1a insert was purified using low melting agarose gel and ligated with the cut pB2LR4 or pB2LR5 vectors to form pB12LR6, pB12LR7, pB12LR8, and pB12LR9 vectors (Figures 3e-3h).

Example 2 – Construction of DNA-launching Platform for wtRNA3 of BMV and for RNA Derivatives Containing Foreign Sequences

Vector pRT101 (Töpfer *et al.*, 1987) was cut with PpuMI and the restriction fragment ends were filled in with Klenow fragment and dNTPs, and cut with BamHI and dephosphorylated. Vector pB3RQ39 (Ishikawa *et al.*, 1997) was cut with SnaBI and BamHI; the B3 fragment was isolated from a low melting agarose gel. This fragment was ligated to the cut pRT101 thereby forming pB3LR10 (Figure 4). The pB3LR15 (Figure 4) that is a pB3LR10 derivative has the ClaI-KpnI fragment replaced with the corresponding fragment from pB3TP8 (Janda *et al.*, 1987).

PCR was performed on pRT101 to amplify an EcoRV and EcoRI fragment. To create a StuI site instead of a PpuMI site, a one nucleotide deletion was performed during the PCR process. The resulting PCR product was cut with EcoRV and EcoRI and inserted into dephosphorylated pRT101 cut with EcoRV and EcoRI to form pRT101LR11. The pRT101LR11 was cut with StuI and BamHI and dephosphorylated. PB3RQ39 was cut with SnaBI and BamHI and a B3 fragment was isolated using a low melting agarose gel. The fragment was then ligated to pRT101LR11 to form pB3LR12 (Figure 4).

Another DNA-launching platform was constructed with wtRNA3 of BMV having a partially doubled CaMV35S promoter; thereby forming pB3LR14 and pB3LR16 (Figure 4).

A DNA-launching platform wherein the BMV RNA3 coat protein was replaced with GUS was also constructed. The pB3MI22 (Ishikawa *et al.*, 1997) was cut with ClaI and StuI and a B3GUS insert was isolated. The pB3LR10 or pB3LR14 DNA-launching constructs were cut with ClaI and StuI and dephosphorylated. The B3GUS fragment was then ligated to the cut pB3LR10 or pB3LR14 thereby forming the pB3GUSLR17 and pB3GUSLR18 DNA-launching constructs (Figure 5).

A DNA-launching platform having a BMV RNA3 with a GUS gene insertion wherein the GUS is downstream of an additional BMV subgenomic promoter was constructed. The pB3LR15 construct was cut with AvaI and the restriction fragment ends were filled in with Klenow fragment and dNTPs. Construct was then cut with ClaI and dephosphorylated. The pB3MI22 was cut with ClaI and StuI and a B3GUS fragment was isolated. The isolated B3GUS fragment was then ligated to the cut pB3LR15 construct to form a new construct of pB3GUSCPLR19 (Figure 5).

A BMV RNA3 based DNA-launching platform with a CP gene inserted downstream of an additional cowpea chlorotic mottle virus (CCMV) subgenomic promoter was constructed. The pB3GUSLR17 construct was cut with StuI and KpnI and dephosphorylated. The pBC3AJ14 (Pacha and Ahlquist, 1991) was cut with NdeI, the ends were blunted by known methods in the art, and then cut with KpnI. A coat protein fragment was then isolated. The coat protein fragment was then ligated to the cut pB3GUSLR17 to form a new construct of pB3GUSCPLR22 (Figure 5).

A DNA-launching platform was constructed having a subgenomic RNA4. The pB4MK2 (M. Kroll, personal communications) was cut with SnaBI and BamHI and a RNA4 fragment was then isolated. The pRT101LR11 construct was cut with StuI and BamHI and dephosphorylated. The fragment and the cut pRT101LR11 construct were then ligated forming pB4LR20 (Figure 5a).

A DNA-launching platform wherein the BMV coat protein was replaced with GFP was constructed. pEGFP (Clontech, CA) was cut with NotI, filled in with Klenow fragment and dNTPs, cut with SalI, and GFP insert was isolated using low-melting agarose gel. The pB3LR15 was cut with SalI and StuI and dephosphorylated. The GFP fragment was then ligated to the cut pB3LR15 thereby forming the pB3GFPLR48 (Figure 6e).

A DNA-launching platform having a BMV RNA3 with a GFP gene insertion wherein the CP is downstream of an additional CCMV subgenomic promoter was constructed. The

pBC3AJ14 (Pacha and Ahlquist, 1991) was cut with NdeI and EcoRI and the ends were blunted by known methods in the art. The coat protein fragment was then isolated and ligated into dephosphorylated and blunted pEGFP cut with NotI and StuI forming pEGFPCPLR49. pEGFPCPLR49 was cut with KpnI and the EGFPCP fragment was isolated using low-melting agarose gel. PB3GFPLR48 was cut with KpnI and dephosphorylated. The EGFPCP fragment was then ligated to the cut pB3GFPLR48 thereby forming the pB3GFPCPLR50 (Figure 6a).

An RNA transcription vector wherein the GFP gene is expressed as a translational fusion with BMV 3a was constructed. The pB3TP10 (Pacha and Ahlquist, 1991) was cut with BamHI and dephosphorylated. The GFP fragment was amplified from pEGFP (Clontech, CA) using PCR and the following primers:

5'GCAGTCGACGGTACCGCGGGCC3'

and

5'CGCGGCCGCGGATCCTGTACAGCTCG3'.

The amplified product was cut with BamHI and purified using low-melting agarose gel. The GFP fragment was ligated to the cut pB3TP10 forming pB3GFPLR47 (Figure 6d). The pB3GFPLR47 was cut with EcoRI and transcribed using T7 RNA polymerase.

An *Agrobacterium* vector containing BMV RNA3 DNA-launching platform was constructed. The pBI101.2LR1 was cut with SmaI and dephosphorylated. The pB3LR15 was cut with PvuII and the B3 fragment was purified using a low-melting agarose gel. The B3 fragment was then ligated to the cut pBI101.2LR1 thereby forming pB3LR42 (Figure 9).

A DNA-launching platform wherein the BMV RNA3 coat protein was replaced with the SHMV (Sunn hemp mosaic virus) coat protein and the GUS gene was inserted downstream of an additional BMV subgenomic promoter was constructed. The pB3RS4 (Sacher *et al.*, 1988) was cut with AvaI, blunted with Klenow fragment and dNTPs, and cut with KpnI. The SHMV coat protein fragment was isolated using a low-melting agarose gel. The pB3GUSLR17 was cut with StuI and KpnI and dephosphorylated. The SHMV coat protein fragment was ligated to the cut pB3GUSLR17 thereby forming pB3GUSCPLR24 (Figure 7).

Other permutations of DNA-launching platforms containing one or more foreign genes and the necessary cis-acting replication signals will be readily appreciated in view of the teachings herein. For examples, see Figures 5-10.

Example 3 – Transfection of *N. tabacum* Protoplasts with DNA-launching Platform

Media:

NT1 Medium (1 liter) was made with Gibco-BRL (MS salt, catalog #11118-031), 3ml of 6% KH_2PO_4, and 0.2 μg/ml 2,4D (final concentration). The pH was adjusted to 5.5-5.7 using KOH, and the resulting mixture was autoclaved.

NT1 Plating Medium (1 liter) was made with NT1 medium and 72.86 g mannitol, the pH was adjusted to 5.5-5.7, and the resulting mixture was autoclaved.

Wash Solution (1 liter) was made with 72.86 g mannitol, the pH was adjusted to 5.5, and the resulting mixture was autoclaved.

Electroporation Buffer was made with 0.8% NaCl, 0.02% KCl, 0.02% KH_2PO_4, 0.11% Na_2HPO_4, and 0.4M mannitol. The pH was adjusted to 6.5, and the resulting mixture was autoclaved.

Enzyme Solution was made with 0.4M mannitol, and 20mM MES. The pH was adjusted to 5.5, and the resulting mixture was autoclaved.

Growth conditions: Cells (*Nicotiana tabacum*) were grown at room temperature in NT1 media with constant shaking (about 200 rpm).

Preparation of cultures for digestion: About 2-3 ml of one-week old suspension culture was subcultured into 50 ml of fresh NT1 media 3 days before the enzyme digestion. The culture was maintained at 28°C under constant shaking.

Enzyme digestion: The enzyme digestion solution was prepared containing the following: 1% cellulysin (Calbiochem) and 0.3% macerase (Calbiochem) in the enzyme solution. The pH was adjusted to 5.5 and filter sterilized.

The cells were centrifuged at 800 rpm for 5 min. The supernatant was discarded. About 40 ml of wash solution was added, cells were resuspended and were centrifuged at 800 rpm for 5 min. The supernatant was discarded. The cells were then resuspended in three volumes of enzyme digestion solution, and incubated for 60 min. at room temperature.

Washing: The cells were transferred into 50 ml plastic tube and centrifuged at 800 rpm for 5 min. The supernatant was discarded. The cells were resuspended in 40 ml of wash solution and centrifuged at 800 rpm for 5 min. The supernatant was discarded. The cells were resuspended in 40ml of electroporation buffer and centrifuged at 800 rpm for 5 min. The supernatant was discarded. The cells were resuspended in four volumes of electroporation buffer.

Electroporation: One ml of cells containing the RNA or DNA inocula was transferred into electroporation cuvettes and placed on ice for 10 min. The cells were then mixed and

electroporated at 500 microF, 250V. The cuvettes were placed on ice for 10 min. The cells were transferred into 10 ml of NT1 plating media.

Incubation and collection of samples: The cells were incubated at room temperature in dark. Samples were collected 24-48 hrs post inoculation.

RNA Analysis: RNA extraction, denaturing 1% agarose gel electrophoresis and Northern blot hybridization were performed by known methods, such as that performed in Rasochova and Miller (1996). Each lane was loaded with equal amounts (approx. 5 μg) of total RNA as determined by spectrophotometry and confirmed by ethidium bromide staining of ribosomal RNA before Northern blot hybridization. 1 X 10^6 cpm/ml of radioactive probe in hybridization buffer was used per hybridization experiment. Replication of RNA3 was confirmed by detection of sgRNA4, thus showing that BMV RNA replication factors 1a and 2a expressed from expression plasmid(s) support efficient replication of RNA3 supplied as *in vitro* transcript (Figure 11) as well as launched from DNA-launching platform (Figure 12).

<u>Example 4 – Production of Transgenic *N. tabacum* Plants</u>

Once a desired molecule was constructed in *E. coli*, the molecule was transferred into *Agrobacterium tumefaciens* by the freeze-thaw method. Vectors pB1LR2, pB2LR4, pB12LR6, and pB12LR7 were all individually used. An *Agrobacterium* strain LBA 4404 containing an appropriate helper Ti plasmid was grown in 5 ml of YEP medium overnight at 28°C. Two ml of the overnight culture were added to 50 ml YEP medium in a 250-ml flask and shaken vigorously (250 rpm) at 28°C until the culture grew to an OD_{500} of 0.5 to 1.0. The culture was chilled on ice. The cell suspension was centrifuged at 3000 g for 5 min. at 4°C. The supernatant solution was discarded. The cells were resuspended in 1 ml of ice-cold 20 mM $CaCl_2$ solution. 0.1-ml aliquots were dispensed into prechilled eppendorf tubes. About 1 μg of plasmid DNA was added to the cells. The cells were frozen in liquid nitrogen. The cells were thawed by incubating the test tube in a 37°C water bath for 5 min. 1 ml of YEP medium was added to the tube and incubated at 28°C for 2-4 h with gentle shaking to allow the bacteria to express the antibiotic resistance genes. The tubes were centrifuged for 30 s and the supernatant solution was discarded. The cells were resuspended in 0.1 ml YEP medium, plated on a YEP agar plate containing selection antibiotic(s), and incubated at 28°C. Transformed colonies appeared in 2-3 days.

In vitro clonal copies of approximately three week old *Nicotina tabacum*, Wisconsin No. 38, were used as the source of explants. Leaf explants were prepared from the second and third fully expanded leaves of *in vitro* cultures. The leaf pieces were cut into 1 cm x 1 cm squares and

placed upon TB1 (plus 2.0 mg/l 6-benzyl-aminopurine, and 0.1 mg/l -naphthalene acetic acid) media for 24 hours at 25°C with a 16 hour photo period.

Agrobacterium tumefaciens strain LBA 4404 containing the preselected binary vector was used for plant transformation. Explants were placed in ~10 ml of overnight grown *Agrobacterium* culture for 30 min. Leaf explants were then blotted on filter paper and placed on TB2 (plus 1.0 mg/l 6-benzyl-aminopurine and 0.1 mg/l -naphthalene acetic acid) media for 4 days, abaxial side down. Explants are then rinsed three times in sterile water, blotted on filter paper, and placed on TB2 media for regeneration with 100 mg/l kanamycin and 400 mg/l carbenicillin at 25°C, 16 hour photo period, abaxial side down. Explants were transferred to fresh TB2 media with 100 mg/l kanamycin and 400 mg/l carbenicillin every 10 to 14 days until plantlets developed. Plantlets typically developed at 10-14 days. Plantlets were cut from the callus and placed on MST media containing 100 mg/l kanamycin and 400 mg/l carbenicillin to induce rooting. Rooted plants were transferred to soil.

TB1 (1 liter) included 4.30 g MS salts, 100 mg myo-inositol, 1.0 ml Nitsch and Nitsch vitamins, 30 g sucrose, 2 mg BAP, 0.10 mg of NAA, and 8g Noble agar. The media was adjusted to a pH 5.7 and autoclaved.

TB2 (1 liter) included 4.30 g MS salts, 100 mg myo-inositol, 1.0 ml Nitsch and Nitsch vitamins, 30 g sucrose, 1.0 mg BAP, 0.10 mg NAA, and 8 g Noble agar. The media was adjusted to pH 5.7 and autoclaved.

MST (1 liter) included 4.30 g MS salts, 1.0 ml Nitsch and Nitsch vitamins, 30 g sucrose, 100 mg myo-inositol, and 8.5 g Difco agar. The media was adjusted to pH 5.7 and autoclaved.

YEP (100 ml) included 1.0g Bacto-peptone, 1.0 g Bacto-yeast extract, and 0.5 g NaCl. The media was autoclaved.

RNA Analysis: Total RNA extraction, denaturing 1% agarose gel electrophoresis and Northern blot hybridization was performed by known methods, such as that performed in Rasochova and Miller (1996). Each lane was loaded with equal amounts (approx. 5 μg) of total RNA as determined by spectrophotometry and confirmed by ethidium bromide staining of ribosomal RNA before Northern blot hybridization. 1 X 10^6 cpm/ml of radioactive probe in hybridization buffer was used per hybridization experiment. Figure 13a shows the successful expression of BMV 1a and 2a mRNA in transgenic *N. tabacum*.

<u>Example 5 – Transfection of Transgenic *N. tabacum* Plants with DNA-launching Platform</u>

Precipitation of DNA onto Microcarriers for Particle Bombardment: (Kikkert, 1993).

Sterilization of Microcarriers: 80 mg of gold microcarriers were resuspended in 1 ml of 70% ethanol, soaked for 15 min., and centrifuged at 13,000 x g for 5 min. The supernatant was carefully removed and discarded. Particles were resuspended in 1 ml of sterile distilled, deionized water and centrifuged at 13,000 x g for 5 min. The supernatant was carefully removed and discarded. Water washing of particles was repeated 2 more times. After final rinse, particles were resuspended in 1 ml of sterile 50% glycerol.

Coating Microcarriers with DNA: The following was sequentially and quickly added: 5μl DNA (1μg/μl), 50μl of 2.5M $CaCl_2$, and 20μl of 0.1M Spermidine.

The mixture was incubated for 10 min. on a vortex shaker at room temperature. Particles were pelleted by centrifugation at 13,000 x g for 5 sec. Supernatant was carefully removed and discarded. Particles were resuspended in 140 μl of 70% ethanol and centrifuged at 13,000 x g for 5 sec. Supernatant was removed and discarded. Particles were resuspended in 140 μl of 100% ethanol and centrifuged at 13,000 x g for 5 sec. Supernatant was removed and discard. Particles were resuspended in 50μl of 100% ethanol.

Young leaves from tobacco plants grown *in vitro* on agar-solidified MS medium containing 30g/liter sucrose, were bombarded with 5-μl aliquots of resuspended DNA-coated particles using a PDS1000He biolistic gun (DuPont) and 1100 psi rupture disks (Bio-Rad).

RNA Analysis: Total RNA extraction, denaturing 1% agarose gel electrophoresis and Northern blot hybridization was performed by known methods, such as that performed in Rasochova and Miller (1996). Each lane was loaded with equal amounts (approx. 5 μg) of total RNA as determined by spectrophotometry and confirmed by ethidium bromide staining of ribosomal RNA before Northern blot hybridization. 1 X 10^6 cpm/ml of radioactive probe in hybridization buffer was used per hybridization experiment. Figure 14a shows that the launched BMV RNA3 replicates efficiently in transgenic plants expressing BMV replication factors 1a and 2a and that the launched RNA3 is unable to replicate in the absence of BMV 1a and/or 2a.

Example 6 – Production of Transgenic *N. benthamiana* Plants

Once a desired molecule was constructed in *E. coli*, the molecule was transferred into *Agrobacterium tumefaciens*. Vectors pB1LR2, pB2LR4, pB12LR6, and pB12LR7 were all individually used. An *Agrobacterium* strain LBA 4404 containing an appropriate helper Ti plasmid was grown in 5 ml of YEP medium overnight at 28°C. Two ml of the overnight culture were added to 50 ml YEP medium in a 250-ml flask and shaken vigorously (250 rpm) at 28°C until the culture grew to an OD_{500} of 0.5 to 1.0. The culture was chilled on ice. The cell suspension was centrifuged at 3000 g for 5 min. at 4°C. The supernatant solution was discarded.

The cells were resuspended in 1 ml of ice-cold 20 mM $CaCl_2$ solution. 0.1-ml aliquots were dispensed into prechilled eppendorf tubes. About 1 μg of plasmid DNA was added to the cells. The cells were frozen in liquid nitrogen. The cells were thawed by incubating the test tube in a 37°C water bath for 5 min. 1 ml of YEP medium was added to the tube and incubated at 28°C for 2-4 h with gentle shaking to allow the bacteria to express the antibiotic resistance genes. The tubes were centrifuged for 30 s and the supernatant solution was discarded. The cells were resuspended in 0.1 ml YEP medium. The cells were plated on a YEP agar plate containing selection antibiotic(s) and incubated at 28°C. Transformed colonies appeared in 2-3 days.

In vitro clonal copies of approximately five-seven weeks old *N. benthamiana* were used as the source of explants. Leaf explants were prepared from the second and third fully expanded leaves of *in vitro* cultures. The leaf pieces were cut into 1cm x 1cm squares and placed upon MS104 media in 100 x 15 mm plates for 24 hours at 23°C with a 16 hour photo period.

Agrobacterium tumefaciens strain LBA 4404 containing the preselected binary vector was used. Explants were placed in ~10ml of overnight grown *Agrobacterium* culture for 30 min. Leaf explants were then blotted on filter paper and placed abaxial side down on MS104 media for 4 days. Explants were then rinsed three times in sterile water, blotted on filter paper, and placed on MS104 media for regeneration with 300 mg/L kanamycin and 400 mg/L carbenicillin. Explants were transferred to fresh MS104 media with 300 mg/L kanamycin and 400 mg/L carbenicillin every 10-14 days until plantlets developed. Plantlets typically developed at 31-50 days. Plantlets were cut from the callus and placed on MST media plus 300 mg/L kanamycin and 400 mg/L carbenicillin to induce rooting. Rooted plants were transferred to soil.

One liter of MS104 included 4.3 g MS salt mixture, 1.0 ml B5 vitamin solution, 30 g sucrose, 1.0 mg BA, 0.1 mg NAA , and 8.0 g Phytagar. The media was adjusted to pH 5.8 and autoclaved.

100 ml of YEP included 1.0 g Bacto-peptone, 1.0 g Bacto-yeast extract, 0.5 g NaCl. The media was autoclaved.

One liter of MST included 4.3 g MS salt mixture, 1.0 ml Nitsch & Nitsch vitamins, 30 g sucrose, 100 mg myo-inositol , and 8.5 g Phytagar. The media was adjusted to pH 5.7 and autoclaved.

RNA Analysis: Total RNA extraction, denaturing 1% agarose gel electrophoresis and Northern blot hybridization was performed by known methods, such as that performed in Rasochova and Miller (1996). Each lane was loaded with equal amounts (approx. 5 μg) of total RNA as determined by spectrophotometry and confirmed by ethidium bromide staining of ribosomal RNA before Northern blot hybridization. 1 X 10^6 cpm/ml of radioactive probe in

hybridization buffer was used per hybridization experiment. Figure 13b shows the successful expression of BMV 1a and 2a mRNA in transgenic *N. benthamiana*.

Example 7 – Transfection of Transgenic *N. benthamiana* Plants

Precipitation of DNA onto Microcarriers for Particle Bombardment: (From Kikkert (1993) "The biolistic PDS 1000/He device", *Plant Cell Tiss. And Org. Cult.* 33:221-226)

Sterilization of Microcarriers: 80 mg of gold microcarriers were resuspended in 1 ml of 70% ethanol, soaked for 15 min., and centrifuged at 13,000 x g for 5 min. The supernatant was carefully removed and discarded. Particles were resuspended in 1 ml of sterile distilled, deionized water and centrifuged at 13,000 x g for 5 min. The supernatant was carefully removed and discarded. Water washing of particles was repeated 2 more times. After final rinse, particles were resuspended in 1 ml of sterile 50% glycerol.

Coating Microcarriers with DNA: To the 50 μl of particles the following was sequentially and quickly added: 5μl DNA (1μg/μl), 50μl of 2.5M $CaCl_2$, and 20μl of 0.1M Spermidine.

The mixture was incubated for 10 min. on a vortex shaker at room temperature. Particles were pelleted by centrifugation at 13,000 x g for 5 sec. Supernatant was carefully removed and discarded. Particles were resuspended in 140 μl of 70% ethanol and centrifuged at 13,000 x g for 5 sec. Supernatant was removed and discarded. Particles were resuspended in 140 μl of 100% ethanol and centrifuged at 13,000 x g for 5 sec. Supernatant was removed and discarded. Particles were resuspended in 50μl of 100% ethanol.

Young leaves from *N. benthamiana* plants grown *in vitro* on agar-solidified MS medium containing 30g/liter sucrose, were bombarded with 5-μl aliquots of resuspended DNA-coated particles using a PDS1000He biolistic gun (DuPont) and 1100 psi rupture disks (Bio-Rad).

RNA Analysis: Total RNA extraction, denaturing 1% agarose gel electrophoresis and Northern blot hybridization was performed by known methods, such as that performed in Rasochova and Miller (1996). Each lane was loaded with equal amounts (approx. 5 μg) of total RNA as determined by spectrophotometry and confirmed by ethidium bromide staining of ribosomal RNA before Northern blot hybridization. 1 X 10^6 cpm/ml of radioactive probe in hybridization buffer was used per hybridization experiment. The launched BMV and RNA 3 showed efficient replication (Figure 14b) in transgenic *N. benthamiana* plants expressing BMV replication factors 1a and 2a and was unable to replicate in the absence of BMV 1a and/or 2a.

Example 8 – Transfection of Transgenic Plants with GUS Containing DNA-launching Platform

Transgenic *N. tabacum* and *N. benthaniana* plants were produced according to the procedures discussed above. The plants were transfected with a DNA-launching platform containing a GUS gene (Figure 5a) by particle bombardment as described in Examples 5 and 7. The plants were incubated for 3-5 days and then assayed for β-glucuronidase (GUS) activity using 1 mg/ml X-Gluc (5-bromo-4-chloro-3-indolyl glucucuronide) as substrate in 0.1M potassium phosphate buffer, pH 7.0, 50 μM potassium ferrocyanide, and 2% Triton® X-100. Following an overnight incubation at 37°C, cells replicating launched RNA3 derivatives and expressing the GUS reporter gene from a subgenomic RNA4 gave rise to blue spots (Figure 15). The launched RNA3 derivative did not replicate and express GUS reporter gene in the absence of BMV RNA replication factors 1a and 2a (*e.g.*, in wt *N. benthamiana* and in wt *N. tabacum*).

Example 9 – Transfection of Transgenic Plants Expressing BMV 1a, 2a, 3a, and CP

A plant is transformed with BMV 1a, 2a, 3a, and CP genes whereby those genes are stably expressed in said plant. This can be done with the procedures outlined above. Any modifications that would be needed would be readily apparent to those skilled in the art in light of the teachings contained herein. A DNA-launching platform encoding an RNA replicon which contains a foreign gene and necessary BMV or CCMV cis-acting replication signals to replicate said replicon is constructed (Figure 10b). Foreign genes to be included in said replicon could include, for example, a *Bacillus thuringiensis* polynucleotide that codes for a *B.t.* protein. Other sequences would include, *e.g.*, sequences that encode herbicide resistance, or any other known sequence that encodes peptides or proteins having desired qualities in plants.

Alternatively, plants can be transformed to express BMV 1a, 2a, 3a, and a TMV coat protein in place of the BMV coat protein. A DNA-launching platform is then made containing one or more foreign genes and the necessary cis-acting replication signals, either BMV or CCMV, and a TMV origin of assembly (Figures 8a, 8b, and 10a). This launching platform provides a distinct advantage as TMV is a rod-shaped virus which has no strict limit on the size of RNA that can be encapsidated. Alternatively, TMV movement protein can be used in place of BMV3a (Figure 7c). Hybrids between tobamo and bromoviruses were shown to be viable (Sacher *et al.*, 1988; De Jong and Ahlquist, 1992).

Other permutations and combinations of genes pretransformed and those included in the DNA-launching platform will readily be appreciated by the skilled artisan in light of the teachings herein. (See, *e.g.*, Figures 8c, 10b, and 10c).

As indicated above, CCMV subgenomic promoter can be substituted for BMV sequences in a desired DNA-launching platform. Because the sequence of CCMV subgenomic promoter differs from the sequence of BMV subgenomic promoter, the probability of recombination that would result in loss of a foreign gene is much lower in a construct having a combination of these two different promoters.

In the above examples, trans-acting components may include, but are not limited to, replication factors, components responsible for cell to cell movement, or components such as the coat protein which may be required for long distance spread, viral proteases responsible for post translational processing, or other known trans-acting functions.

Example 10 – Transfection of *N. tabacum* Protoplasts with GUS Containing DNA-Launching Platforms

N. tabacum protoplasts isolated using the above described methods were inoculated by electroporation with DNA-launching platforms for BMV RNA3 derivatives in the presence or absence of 1a and 2a expression plasmids. BMV RNA3 derivatives contained the GUS gene in place of the coat protein ORF (Figure 5a) (these were inoculated with or without coat protein expression plasmid, Figure 5b), or had the BMVCP gene translated from an additional subgenomic RNA driven from BMV or CCMV subgenomic promoter (Figures 5c and 5d), or had the SHMV coat protein translated from an additional BMV subgenomic RNA (Figure 7b). Protoplasts were collected by centrifugation (800 rpm, 5 min.) 24 hours post inoculation. The chemiluminescent GUS assay was performed using GUS-Light™ (Tropix, MA) according to manufacturer's instructions. Protein concentrations were determined using the Bio-Rad protein kit (Bio-Rad Laboratories, Hercules, CA). The GUS values, determined by luminometer, were adjusted to the same total protein concentration. Figures 16a and 16b show successful GUS expression in protoplasts in the presence of trans-acting BMV replication factors 1a and 2a.

Example 11 – Transfection of *N. tabacum* Protoplasts with GFP Containing DNA-Launching Platform

N. tabacum protoplasts isolated by using the above described methods were transfected by electroporation with expression plasmids for trans-acting BMV replication factors 1a and 2a and with DNA-launching platforms for RNA3 derivatives having the GFP gene in place of BMV coat protein ORF (Figure 6e), the CP gene translated from an additional subgenomic RNA (Figure 6a) or with an RNA transcript having the GFP expressed as a fusion protein with BMV 3a ORF (Figure 6d). Protoplasts were incubated for 24 hrs and examined for GFP expression

using a fluorescent microscope. Figure 18 shows the successful expression of GFP in protoplasts.

Example 12 – Transfection of (1a + 2a)-Transgenic Plants with BMV RNA3-Based DNA-Launching Platform Containing GFP

N. benthamiana plants were transfected using a particle bombardment as described above with a DNA-launching platform for BMV RNA3 having the GFP gene in place of BMV coat protein (Figure 6e). The GFP expression was determined 24 hrs post inoculation using a fluorescent microscope. Figure 17 shows the successful expression of GFP in (1a + 2a)-transgenic *N. benthamiana*.

Example 13 – Transfection of (1a + 2a)-Transgenic *N. benthamiana* with BMV RNA3 DNA-Launching Platform Using *Agrobacterium*

N. benthamiana plants were inoculated with BMV RNA3 DNA-launching platform using *Agrobacterium tumefaciens*. Once the desired construct (pB3LR42) was obtained in *E. coli* it was transferred to *A. tumefaciens* strain LBA4404 using a thaw-freeze method as described above. The *Agrobacterium* was grown overnight in 28°C under constant shaking. A single lower leaf of *N. benthamiana* were punctured with a needle multiple times and submerged in *Agrobacterium* culture. The plants were grown at 23°C with a 16 hr photoperiod. The inoculated leaves were harvested 14 days post-inoculation. The total RNA extraction and northern blot hybridization were performed as described above. Figure 19 shows replication of launched BMV RNA3 in inoculated (1a + 2a)-transgenic *N. benthamiana*.

Example 14 – Transfection of (1a + 2a)-Transgenic Plants with BMV RNA3-Based DNA-Launching Platform Containing GUS and SHMV Coat Protein

N. benthamiana plants were transfected using a particle bombardment as described above with a DNA-launching platform for BMV RNA3 wherein the BMV coat protein was replaced with the SHMV coat protein (Sunn-hemp mosaic virus) and the GUS gene was inserted downstream of an additional BMV subgenomic promoter (Figure 7b). The GUS expression was determined by histochemical GUS assay described above. Figure 20 shows the successful expression of GUS in (1a + 2a)-transgenic plants.

Example 15 – Movement of Launched BMV RNA 3

F1 progeny plants from self-fertilized (1a+2a)-transgenic *N. benthamiana* BP14 were inoculated with BMV RNA3 DNA launching platform using *Agrobacterium tumefaciens*. Seedlings were germinated on *Smurf* media containing Kanamycin. Plants were grown at 23°C with a 16 hr photoperiod. Once the desired construct (pB3LR42) was obtained in *E. coli* it was transferred to *A. tumefaciens* strain LBA4404 using a thaw-freeze method as described above. The *Agrobacterium* was grown overnight at 28°C under constant shaking. A single lower leaf of *N. benthamiana* was punctured with a needle multiple times and submerged in *Agrobacterium* culture. The inoculated, middle, and upper leaves were harvested 14 days post-inoculation. Total RNA extraction and northern blot hybridization were performed as described above. RNA3 replication was detected in all leaves tested (Fig. 21). It shows that BMV RNA3 is able to replicate, move cell-to-cell and spread long distance in (1a+2a)-transgenic plants.

Example 16 – Transfection of Progeny From (1a+2a)-Transgenic *N. benthamiana* With BMV RNA3 DNA-Launching Platform

Progeny plants from self-fertilized (1a+2a)-transgenic *N. benthamiana* (designated BP14) were inoculated with BMV RNA3 DNA-launching platform using *Agrobacterium* as described in Example 13. Control plants (non-transgenic *N. benthamiana*) were inoculated with the sap from BMV infected barley using inoculation buffer composed of 50mM $NaPO_4$, pH7.0, and 1% celite. Root samples were harvested 6 weeks post inoculation. RNA extraction and northern blot hybridization were performed as described above. Figure 22 shows that BMV RNA3 replicated to very high levels in roots. In some (1a+2a)-transgenic plants (Figure 22, lanes 2, 5, 6, 7, 8, 10) replication of launched RNA3 dramatically exceeded replication of wild-type BMV in non-transgenic *N. benthamiana* plants (Figure 22, lane 1). This shows that this system can be used for delivery of RNA, proteins, peptides or other compounds to roots and enables testing of such compounds for various activities, for example, activities directed against root parasites. For example, proteins with anti-nematode activities can be inserted into RNA3 DNA-launching platform using the above described strategies and expressed in roots upon RNA3 replication. Such proteins can be engineered to be expressed in the cytoplasm or alternatively secreted into the surrounding soil.

Example 17 – Barley Stripe Mosaic Virus

Barley stripe mosaic virus (BSMV) has a tripartite genome (RNA alpha, beta, and gamma). These genomic RNAs have an m7Gppp cap at the 5′ end and a t-RNA like structure at the 3′ end (Jackson and Hunter, 1989).

A DNA-launching plasmid for BSMV RNA alpha, RNA beta, and RNA gamma containing BSMV RNA cDNA is constructed by precisely fusing at its 5′ end to a DNA-dependent RNA polymerase promoter and to a self-cleaving ribozyme at its 3′ end. A polyadenylation signal may be also included. Alternatively, a convenient restriction site may be engineered at the 3′ end of viral cDNAs. Foreign genes or sequences may be expressed in several ways. For example, DNA-launching plasmids based on BSMV RNA beta may contain a foreign gene or sequence expressed in place of ORF beta a.

Transgenic plants having one or more trans-acting factors fused to the DNA-dependent RNA polymerase promoter and terminator are obtained. Such trans-acting factors may include parts of the viral RNA replicase (ORFs alpha a and/or gamma a) or other trans-acting factors. The trans-acting factors are stably expressed in the plant cell or their expression may be induced if an inducible promoter is used. Cis-acting sequences necessary for BSMV RNA replication are removed from transgenes. Alternatively, the full-length RNA alpha is expressed from the chromosome. Alternatively, ORF gamma a including the 5′ untranslated region and ORF gamma b from a seed transmitted strain, such as ND18, are also expressed (Edwards, 1995).

A DNA-launching plasmid is constructed containing the DNA-dependent RNA polymerase promoter precisely fused to the 5′ end of the BSMV RNA beta, cis-acting elements important for BSMV RNA beta life cycle, such as the 5′ and 3′ ends, the intercistronic region between the beta a and beta b ORFs (Zhou and Jackson, 1996) and a foreign gene or sequence in place of ORF beta a (coat protein) which is dispensable for BSMV replication and movement (Petty and Jackson, 1990). Such DNA-launching plasmids may lack the internal poly(A) region as this region is dispensable for replication and contain a ribozyme or a convenient restriction site at the 3′ end of the modified viral RNA. Alternatively, a DNA-launching plasmid is constructed from RNA gamma in which ORFs gamma a and/or gamma b are replaced with foreign genes or sequences which may also include the triple gene block genes (ORFs beta b, beta c, and beta d) or a heterologous movement protein (TMV 30K, RCNMV 35K).

Example 18 – Tobacco Mosaic Virus

Tobacco mosaic virus (TMV) has a single-stranded positive sense RNA genome. The 5′ end has an m7Gppp cap and the 3′ end contains a t-RNA like structure.

A DNA-launching plasmid is constructed based on TMV RNA containing TMV cDNA precisely fused at its 5′ end to a DNA-dependent RNA polymerase promoter and at its 3′ end to a self-cleaving ribozyme. A polyadenylation signal may be also included. Alternatively, a convenient restriction site may be engineered at the 3′ end. Foreign gene may be expressed from an additional subgenomic RNA by including an additional subgenomic RNA promoter on the (-) strand.

Transgenic plants are obtained having one or more trans-acting factors fused to the DNA-dependent RNA polymerase promoter and terminator. Such factors may include the viral replicase (126K/183K), movement protein (30K), or coat protein (17.6K). At least one cis-acting sequence necessary for TMV RNA replication is removed from transgenes. The trans-acting factors are stably expressed in the plant cell or their expression may be induced if an inducible promoter is used.

A DNA-launching plasmid is constructed containing the DNA-dependent RNA polymerase promoter precisely fused to the 5′ end of the TMV cDNA, cis-acting elements important for the TMV life cycle, such as the 5′ and 3′ ends, origin of assembly, etc., at least one foreign gene or sequence in place of the trans-acting factor that is expressed from the chromosome, and a ribozyme or a convenient restriction site at the 3′ end. Alternatively, the foreign gene sequence can be expressed from an additional subgenomic RNA promoter and the sequence coding for the trans-acting factor that is expressed from the transgene can be deleted from the DNA-launching plasmid. Preferably, if the viral replicase proteins are expressed in transgenic plants, the DNA-launching plasmid will have a deletion of nucleotides 3420-4902, which appears to be a region that inhibits replication in trans. (Lewandowski *et al.*, 1998).

Example 19 – Potato Virus X

Potato virus X (PVX) has a single-stranded positive sense RNA genome. The 5′ end has an m7Gppp cap and the 3′ end is polyadenylated. A full-length cDNA clone of PVX has been constructed and infectious RNA transcripts obtained (Hemenway *et al.*, 1990).

A DNA-launching plasmid is constructed based on PVX RNA containing PVX cDNA precisely fused at its 5′ end to a DNA-dependent RNA polymerase promoter and having a polyadenylation site at its 3′ end. A convenient restriction site may also be included at the 3′ end. A foreign gene may be expressed from an additional subgenomic RNA.

Transgenic plants are obtained having one or more trans-acting factors fused to the DNA-dependent RNA polymerase promoter and terminator. Such factors may include the viral RNA polymerase gene (ORF1-147K), coat protein (ORF5-21K), or triple gene block (ORF2-

25K, ORF3-12K, ORF4-8K). The triple gene block genes can be expressed individually. Alternatively, they can be expressed as negative sense transcripts from which plus sense subgenomic RNA for ORFs 2, 3, and 4 can be transcribed by the viral replicase. Such transgene will have a DNA-dependent RNA polymerase promoter fused to sequence of ORFs 2, 3, and 4 in the minus sense orientation and the transcribed sequence will include a subgenomic RNA promoter. At least one cis-acting sequence necessary for PVX RNA replication is removed from transgenes. The trans-acting factors are stably expressed in the plant cell or their expression may be induced if an inducible promoter is used.

A DNA-launching plasmid is constructed containing the DNA-dependent RNA polymerase promoter precisely fused to the 5′ end of the PVX genome, cis-acting elements important for PVX life cycle, such as the 5′ and 3′ ends, origin of assembly, etc., at least one foreign gene or sequence in place of the trans-acting factor that is expressed from the chromosome and a polyadenylation signal. Alternatively, the foreign gene sequence can be expressed from an additional subgenomic RNA promoter and the sequence coding for the trans-acting factor that is expressed transgenically can be deleted from the DNA-launching plasmid.

Alternatively, a DNA-launching plasmid is constructed having a DNA-dependent RNA polymerase promoter, polyadenylation site, and the PVX cDNA sequence in which the ORF2 (25K) is replaced with a foreign gene or sequence. Alternatively, the ORF2 is deleted and the foreign gene is expressed from an additional subgenomic RNA promoter. Such a DNA-launching plasmid is inoculated to transgenic plants expressing movement protein from heterologous virus, such as tobacco mosaic virus (TMV 30K), tomato mosaic virus (ToMV 30K), or red clover necrotic mosaic virus (RCNMV 35K).

Example 20 – Flock House Virus

Flock house virus (FHV) has a genome consisting of two single stranded RNAs. RNA1 encodes protein A, involved in RNA replication, and protein B that is translated from sg RNA3 and is dispensable for RNA replication. RNA2 encodes virion capsid precursor protein alpha. FHV is infectious to insect, plant, mammalian, and yeast cells (Selling *et al.*, 1990; Price *et al.*, 1996).

A DNA-launching plasmid is constructed for FHV RNA1 and RNA2 containing FHV RNA cDNA precisely fused at its 5′ end to a DNA-dependent RNA polymerase promoter and at its 3′ end to a self-cleaving ribozyme. A polyadenylation signal may be also included. Alternatively, a convenient restriction site may be engineered at the 3′ end. Foreign genes or sequences may be expressed in several ways. For example, DNA-launching plasmids based on

FHV RNA1 may contain a foreign gene or sequence expressed from subgenomic RNA3 as ORF B replacement or as a translational fusion with ORF B. Alternatively, a foreign gene may be expressed from an additional sg RNA. DNA-launching plasmids based on FHV RNA2 may contain a foreign gene(s) or sequence(s) expressed as a part of polyprotein alpha. Foreign gene(s) in such construct may include sequences necessary for polyprotein clevage. DNA-launching plasmids will preferably also express a movement protein of a heterologous plant virus, such as 30K of TMV or 35K of RCNMV. Alternatively, DNA-launching plasmids will be inoculated onto transgenic plants expressing such movement protein.

Transgenic plants are obtained having one or more trans-acting factors fused to the DNA-dependent RNA polymerase promoter and terminator. Such factors may include protein A or capsid protein precursor alpha, and preferably will also include a movement protein from a plant virus, such as 30K of TMV or 35K of RCNMV. Trans-acting factors are stably expressed in the plant cell or their expression may be induced if an inducible promoter is used. Transgenically expressed trans-acting factors preferably lack at least one cis-acting factor which is necessary for their replication, such as the 5′ and/or 3′ end.

A DNA-launching plasmid is constructed based on FHV RNA1 or FHV RNA2 containing a DNA-dependent RNA polymerase promoter precisely fused to the 5′ end of RNA1 (or RNA2), cis-acting elements important for FHV RNA1 (or RNA2) replication, such as the 5′ and 3′ ends, at least one foreign gene or sequence and a self-cleaving ribozyme at the 3′ end. Polyadenylation signal may also be included. Alternatively, a convenient restriction site may be engineered at the 3′ end of the modified viral RNA sequence of the DNA-launching plasmid. DNA-launching plasmids based on FHV RNA1 may contain a foreign gene or sequence in place of ORF A. Alternatively, the ORF A may be deleted and the foreign gene may be expressed from subgenomic RNA3, for example as an ORF B replacement or as a translational fusion with ORF B. Alternatively, DNA-launching plasmid may contain two exogenous RNA sequences, one in the place of ORF A and the other expressed from the subgenomic RNA3. DNA-launching plasmids based on FHV RNA2 may contain a foreign gene(s) or sequence(s) in place of ORF alpha or expressed as a part of polyprotein alpha. Foreign gene(s) in such a construct may include sequences necessary for polyprotein clevage.

<u>Example 21 – Tomato Spotted Wild Virus</u>

Tomato spotted wild virus (TSWV) is a tripartite (RNA L, M, S), negative sense and ambisense, single stranded RNA virus.

Transgenic plants are obtained having one or more trans-acting factors fused to the DNA-dependent RNA polymerase promoter and terminator. Such factors include the putative TSWV polymerase gene (ORF L), ORF N, and possibly other trans-acting factors (NSm or NSs). At least one cis-acting sequence, such as 5′ and/or 3′ ends, which are necessary for TSWV RNA replication are removed from the transgene. Trans-acting factors are stably expressed in the plant cell or their expression may be induced if an inducible promoter is used.

A DNA-launching plasmid is constructed based on TSWV RNA M in which the G1 and G2 coding sequences are replaced with at least one foreign gene or sequence. Such DNA-launching plasmid contains a DNA-dependent RNA polymerase promoter and TSWV RNA M cDNA fused to the self-cleaving ribozymes at the 5′ and 3′ ends. Alternatively, a DNA-launching plasmid is constructed based on TSWV RNA S in which the N coding region is replaced with a foreign gene or sequence.

Example 22 – Barley Mild Mosaic Virus

Genome of barley mild mosaic virus (BaMMV) consists of two positive sense, single-stranded, 3′-polyadenylated RNAs. The RNA1 encodes proteins related to the potyviral P3, 6K1, CI, 6K2, NIa-VPg, NIa-Pro, NIb and capsid protein (Kashiwazaki *et al.*, 1990). The RNA2 encodes P1 and P2 protein (Kashiwazaki *et al.*, 1991). The P1 protein is related to the potyviral HC-Pro and the P2 protein is important for fungal transmission. An isolate was obtained containing a deletion in the P2 protein (Timpe and Kuhne, 1995) thus indicating that P2 is dispensable for viral RNA replication.

A DNA-launching plasmid is constructed for BaMMV RNA1 and RNA2 containing BaMMV RNA cDNA precisely fused at its 5′ end to a DNA-dependent RNA polymerase promoter and a polyadenylation site at its 3′ end. Foreign genes or sequences may be expressed in several ways. For example, DNA-launching plasmids based on BaMMV RNA2 may contain a foreign gene or sequence expressed as a part of polyprotein which can be cleaved and a foreign protein can be released.

Transgenic plants are obtained having the BaMMV RNA1 cDNA lacking the 5′ and 3′ ends fused to the DNA-dependent RNA polymerase promoter and terminator.

A DNA-launching plasmid is constructed based on BaMMV (isolate M) RNA2. Such plasmid contains a DNA-dependent RNA polymerase promoter precisely fused to the 5′ end of RNA2, RNA2 cis-acting replication signals located in the 5′ and 3′ ends, P1 ORF and a foreign gene in place of P2 ORF or expressed as a part of P1/P2 polyprotein which can be cleaved and a foreign protein can be released.

The contents of all references cited throughout are incorporated herein by this reference to the extent they are not inconsistent with the disclosure, teachings, and principles of the subject invention.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.

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SEQUENCE LISTING

<110> WISCONSIN ALUMNI RESEARCH FOUNDATION
Street Address: 614 Walnut Street
City: Madison
State: Wisconsin
Country: US
ZIP: 53705
Phone number: (608) 265-2135 Fax: (608) 263-1064

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Claims

1. A DNA-launching platform comprising:

a) a polynucleotide molecule encoding a modified viral RNA molecule; and

b) a DNA dependent RNA polymerase promoter.

2. The DNA-launching platform of claim 1 further comprising a sequence encoding at least one cis-acting element.

3. The DNA-launching platform of claim 1 further comprising a ribozyme sequence.

4. The DNA-launching platform of claim 1 further comprising a termination sequence.

5. The DNA-launching platform of claim 1 further comprising a restriction site.

6. The DNA-launching platform of claim 1 wherein said modified RNA molecule comprises an exogenous RNA segment.

7. The DNA-launching platform of claim 1 wherein said DNA dependent RNA polymerase promoter is capable of functioning in a plant cell.

8. A method of genotypically or phenotypically modifying one or more cells comprising the following steps:

a) obtaining a DNA-launching platform comprising a polynucleotide molecule encoding a modified viral RNA; and

b) transfecting said one or more cells with said DNA-launching platform, wherein said polynucleotide molecule is transcribed thereby forming a replicatable RNA transcript.

9. The method of claim 8 further comprising pre-transforming said cell with at least one polynucleotide molecule encoding at least one trans-acting factor.

10. The method of claim 8 further comprising introducing a trans-acting factor.

11. The method of claim 10 wherein said introducing a trans-acting factor comprises co-transfection of an expression plasmid comprising a nucleotide sequence encoding said trans-acting factor.

12. The method of claim 10 wherein said introducing a trans-acting factor comprises co-transfection of an RNA transcript encoding said trans-acting factor.

13. The method of claim 10 wherein said trans-acting factor is stably expressed.

14. The method of claim 8 wherein said modified viral RNA comprises an exogenous RNA segment.

15. The method of claim 8 wherein said DNA-launching platform comprises a ribozyme sequence.

16. The method of claim 8 wherein said DNA-launching platform comprises a promoter.

17. The method of claim 8 wherein said DNA-launching platform comprises a termination sequence.

18. The method of claim 8 wherein said DNA-launching platform comprises a restriction site.

19. The modified cell produced by the method of claim 8.

20. A method of producing a plant or plant tissue comprising at least one genotypically or phenotypically modified cell, said method comprising transfecting cells of said plant or plant tissue with a DNA-launching platform, wherein said DNA-launching platform comprises a polynucleotide encoding a modified RNA molecule, such that said polynucleotide molecule is transcribed to form a replicatable RNA transcript.

21. The method of claim 20 wherein said modified RNA molecule comprises an exogenous RNA segment.

22. The method of claim 20 wherein said DNA-launching platform comprises a ribozyme sequence.

23. The method of claim 20 wherein said DNA-launching platform comprises a promoter.

24. The method of claim 20 wherein said DNA-launching platform comprises a termination sequence.

25. The method of claim 20 wherein said DNA-launching platform comprises a restriction site.

26. A method of producing a genotypically or phenotypically modified plant comprising obtaining at least one modified cell produced by the method of claim 8; and subjecting said modified cell to conditions whereby a plant is regenerated therefrom.

27. A plant produced by the method of claim 26.

28. A plant descended from the plant of claim 27.

29. The method of claim 20, wherein said plant or plant tissue comprises one or more cells transformed with a polynucleotide molecule encoding at least one trans-acting factor, wherein said polynucleotide molecule is expressed.

30. The method of claim 29, wherein said modified viral RNA molecule is capable of replication only in said one or more cells transformed with a polynucleotide molecule encoding at least one trans-acting factor.

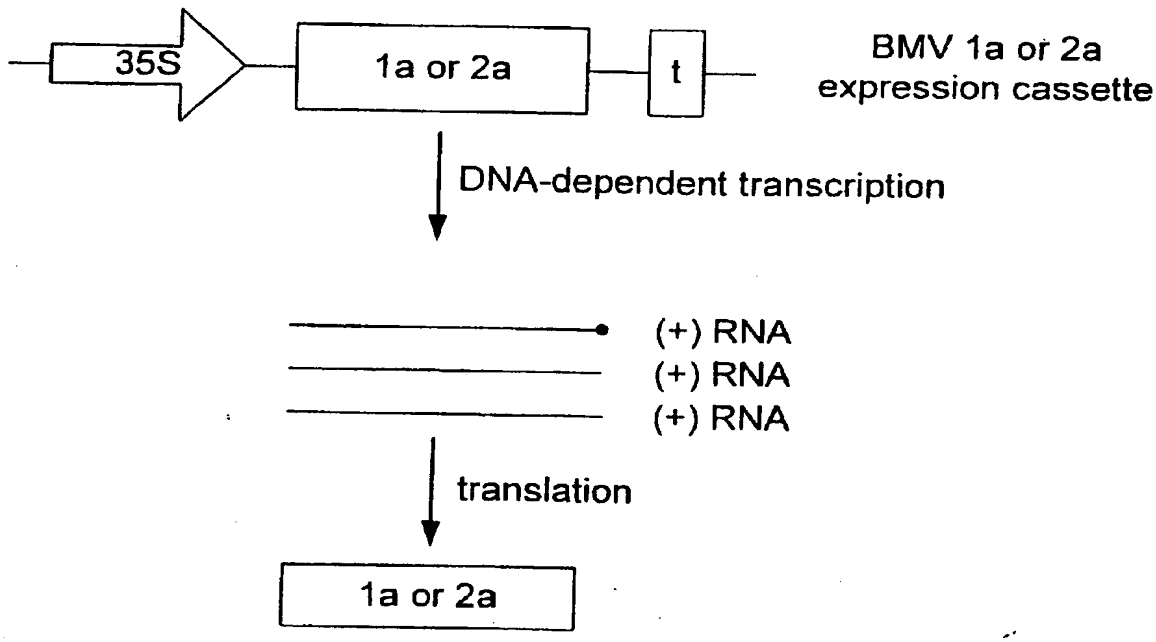

35S
1a or 2a
t
BMV 1a or 2a
expression cassette
DNA-dependent transcription
(+) RNA
(+) RNA
(+) RNA
translation
1a or 2a

FIG. 1

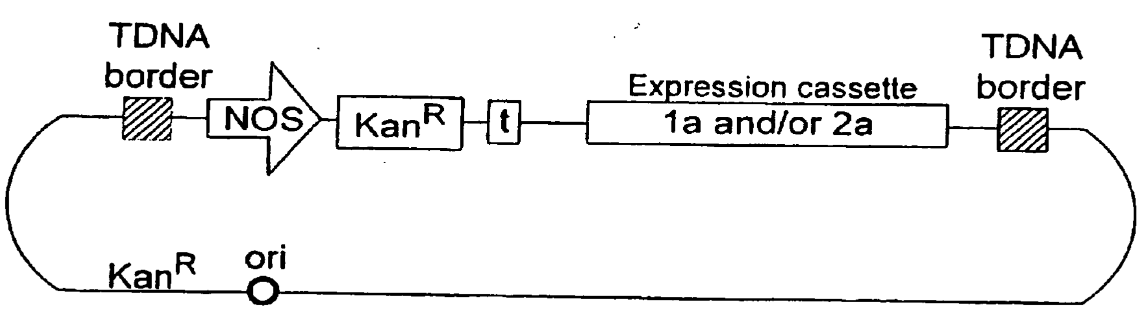

TDNA
border
NOS
Kan^R
t
Expression cassette
1a and/or 2a
TDNA
border
Kan^R
ori

FIG. 2

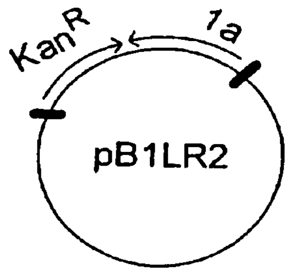


FIG. 3A

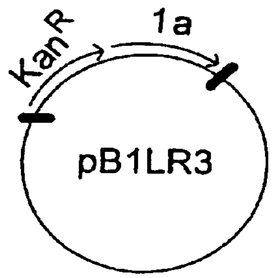


FIG. 3B

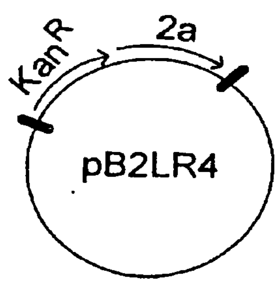


FIG. 3C

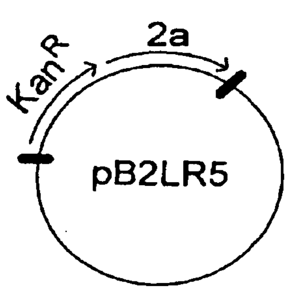


FIG. 3D

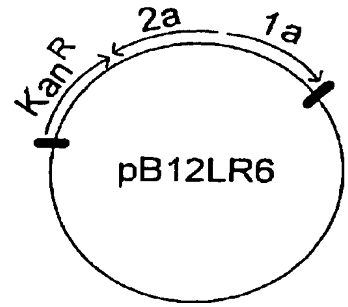


FIG. 3E

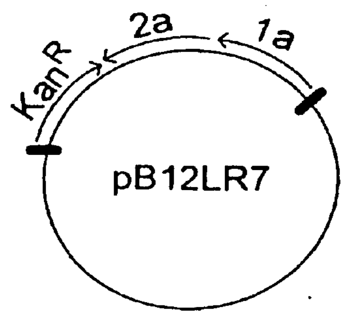


FIG. 3F

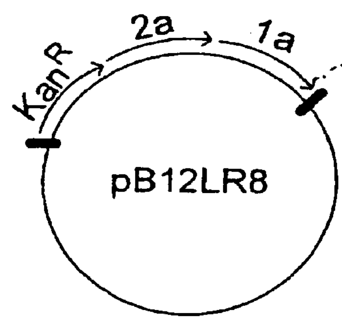


FIG. 3G

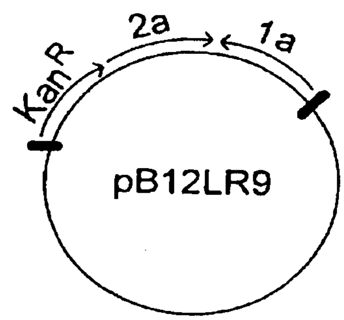
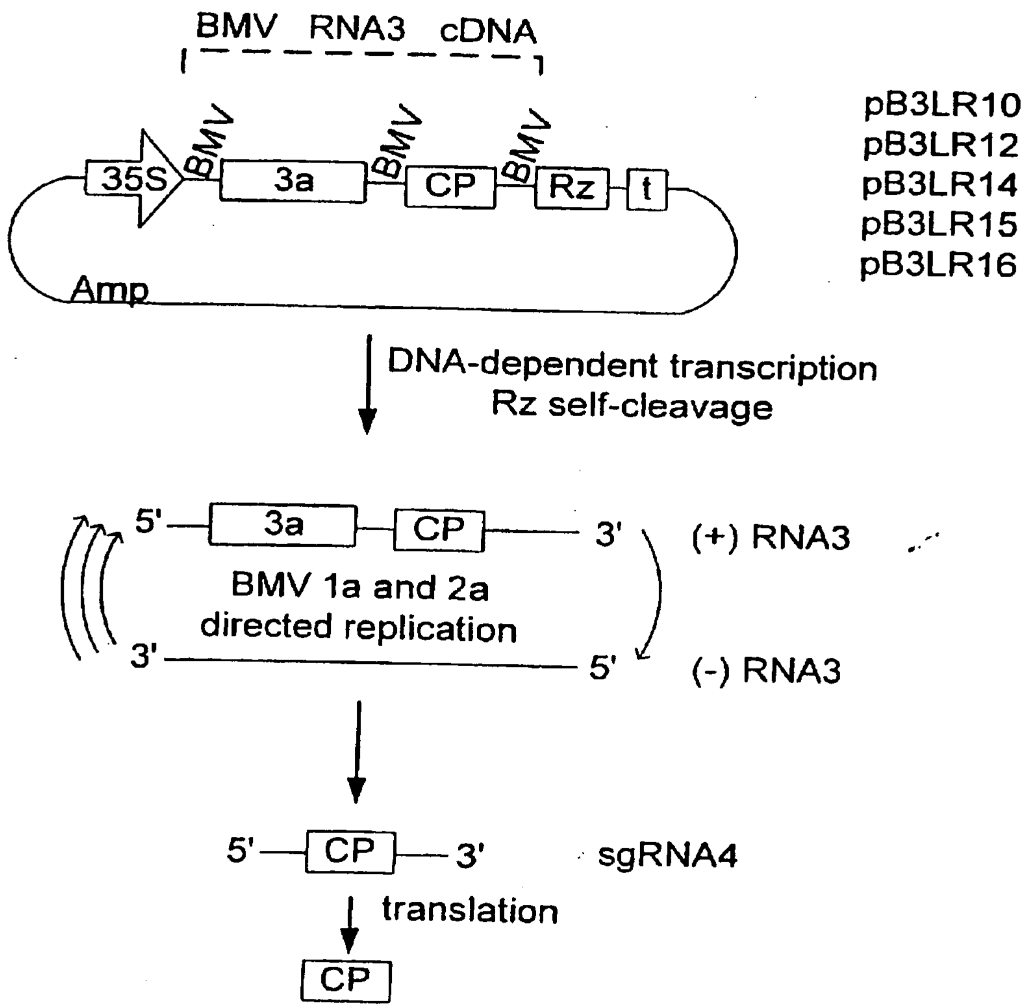


FIG. 3H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35S
BMV
3a
BMV
CP
BMV
Rz
t
Amp
pB3LR10
pB3LR12
pB3LR14
pB3LR15
pB3LR16
DNA-dependent transcription
Rz self-cleavage
5'
3a
CP
3'
(+) RNA3
BMV 1a and 2a
directed replication
3'
5'
(-) RNA3
5'
CP
3'
sgRNA4
translation
CP

FIG. 4

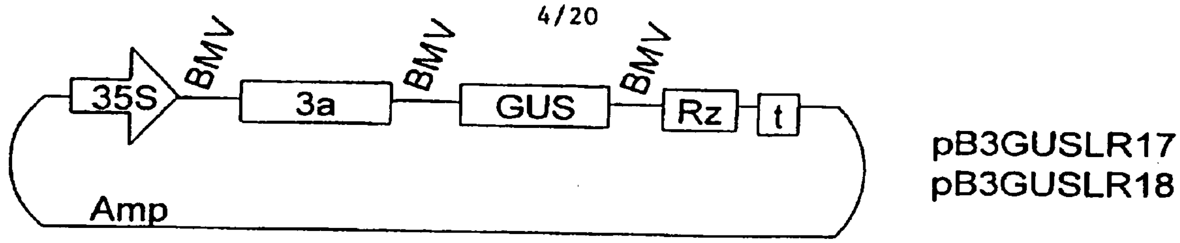

FIG. 5A

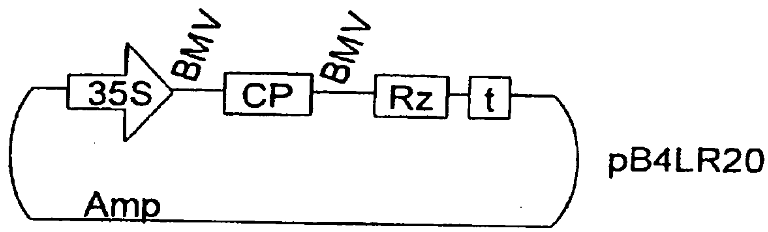

FIG. 5B

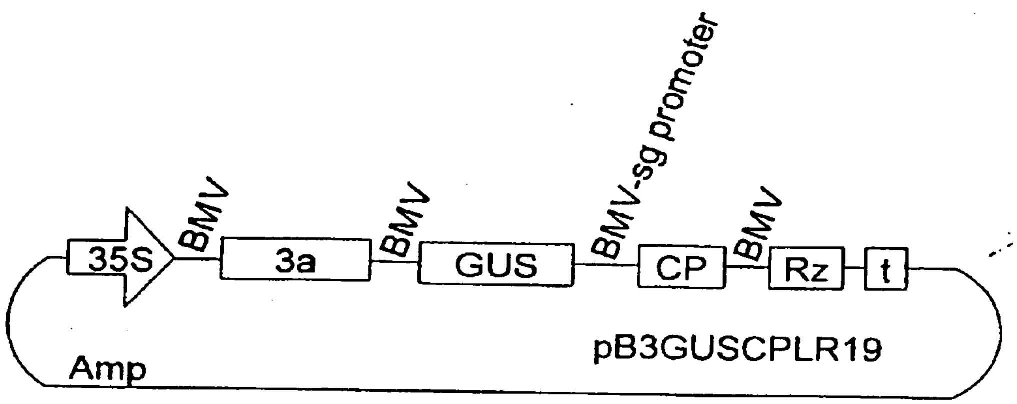

FIG. 5C

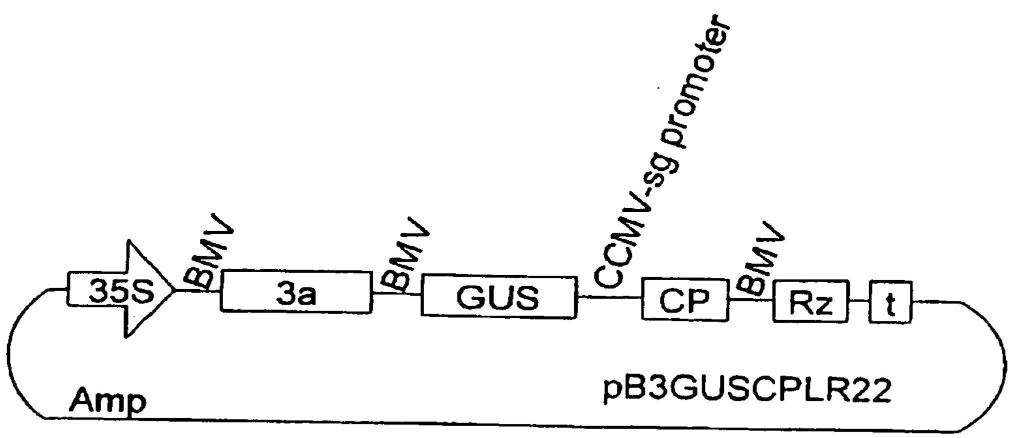

FIG. 5D

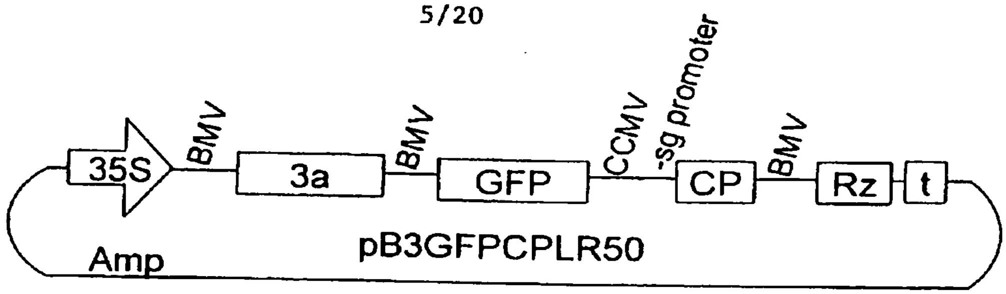

FIG. 6A

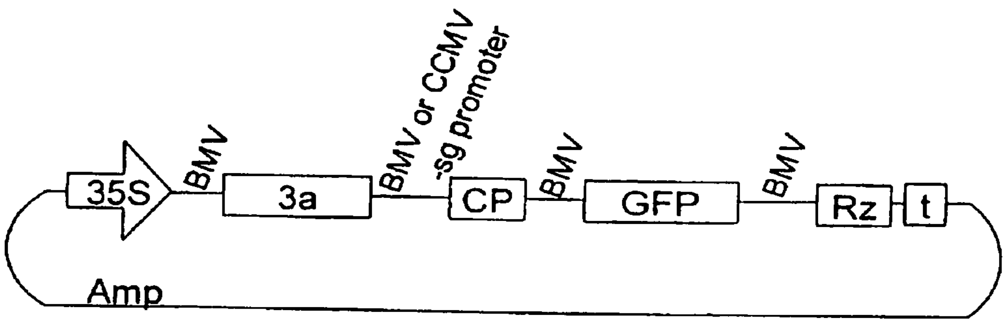

FIG. 6B

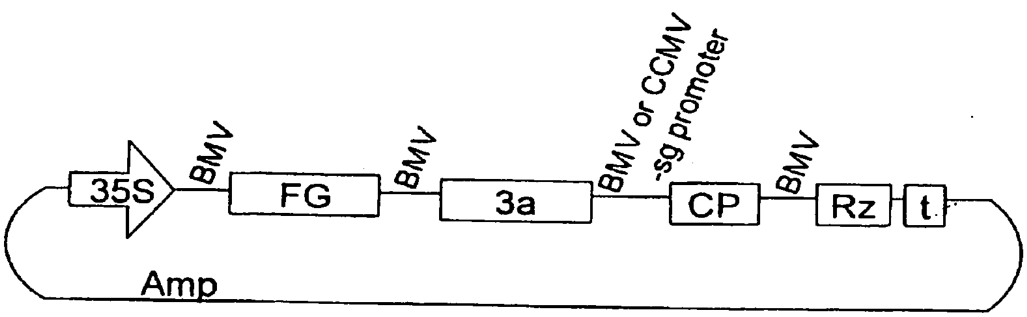

FIG. 6C

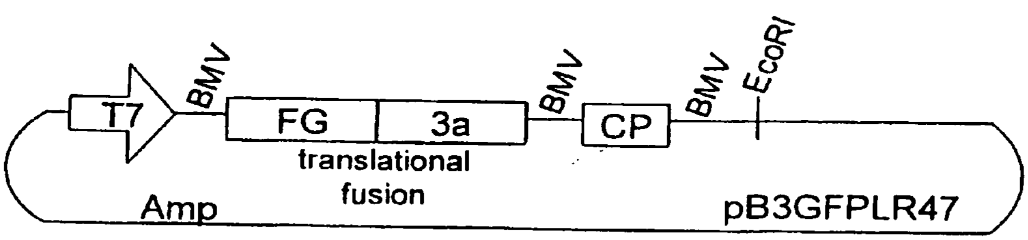

FIG. 6D

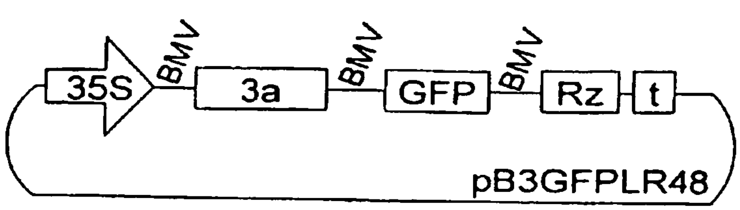

FIG. 6E

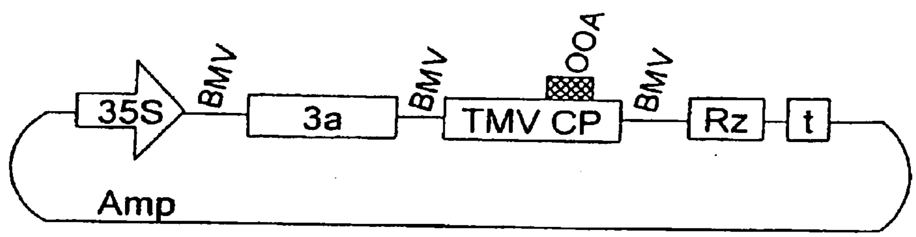

FIG. 7A

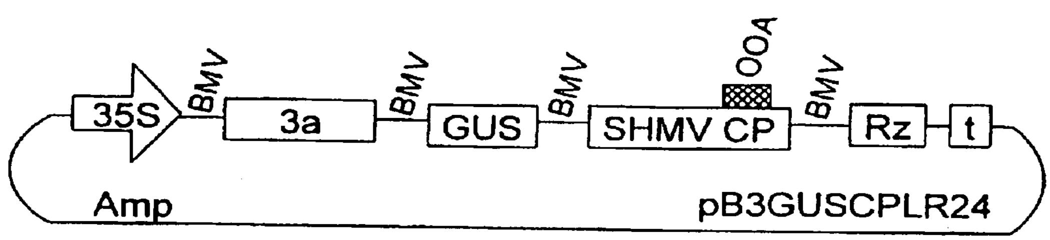

FIG. 7B

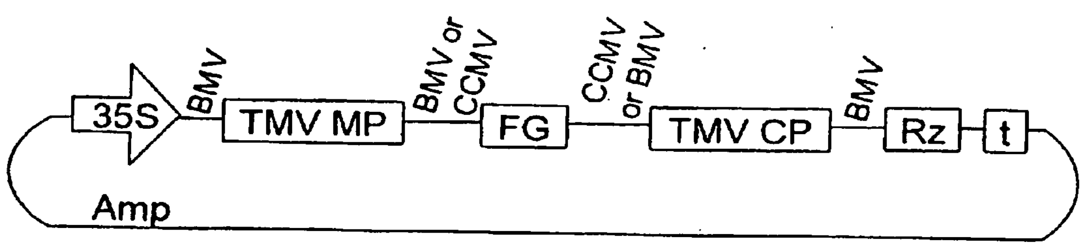

FIG. 7C

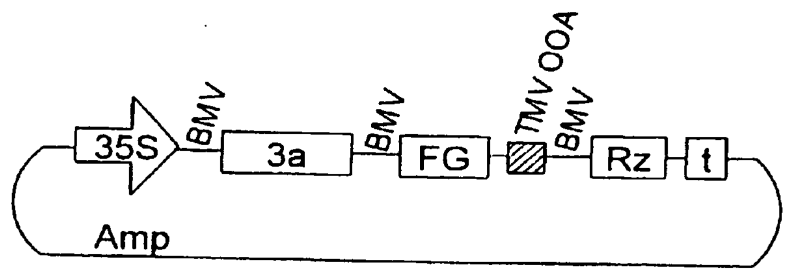

FIG. 8A

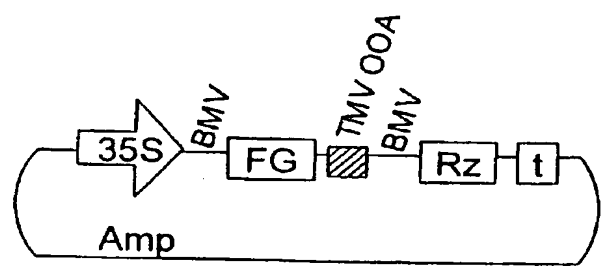

FIG. 8B

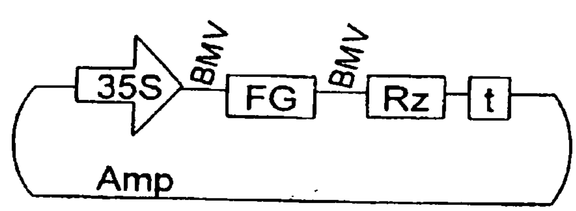

FIG. 8C

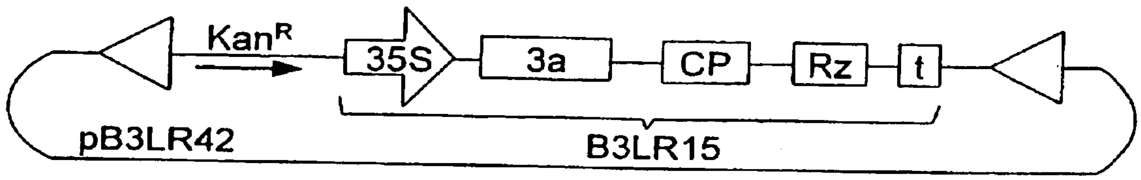

FIG. 9

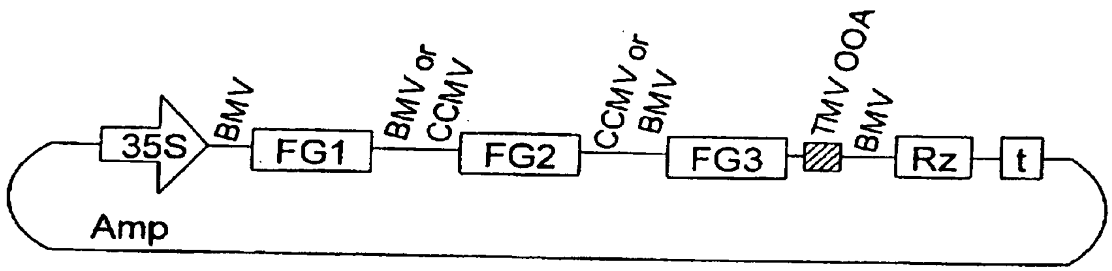

FIG. 10A

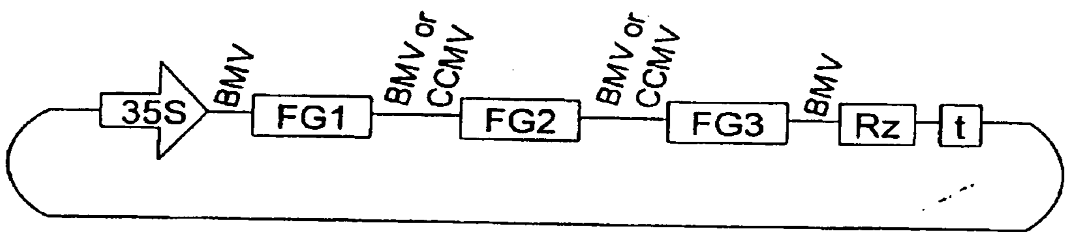

FIG. 10B

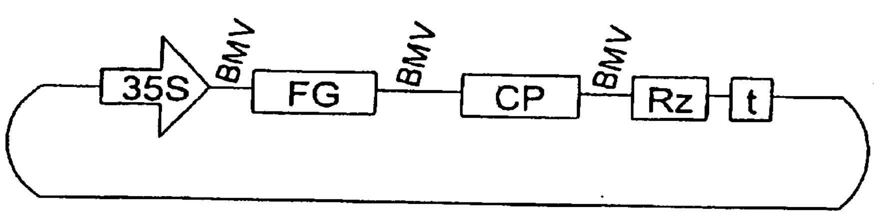

FIG. 10C

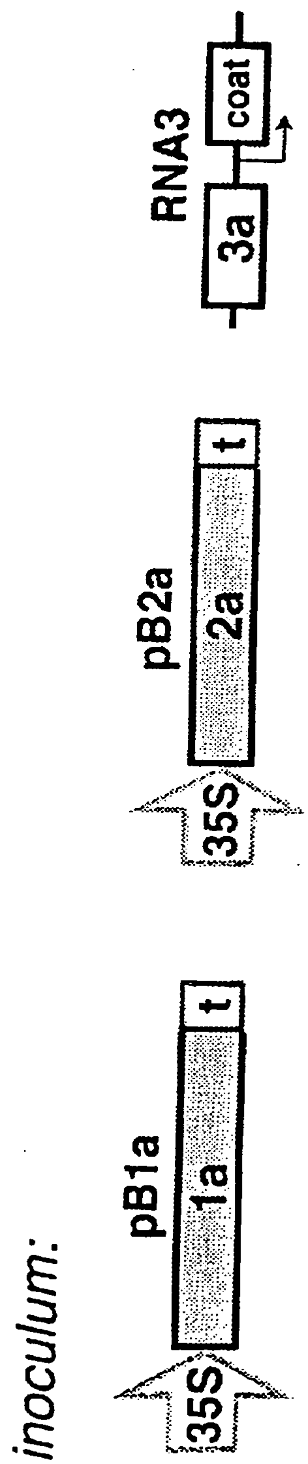

FIG. 11A

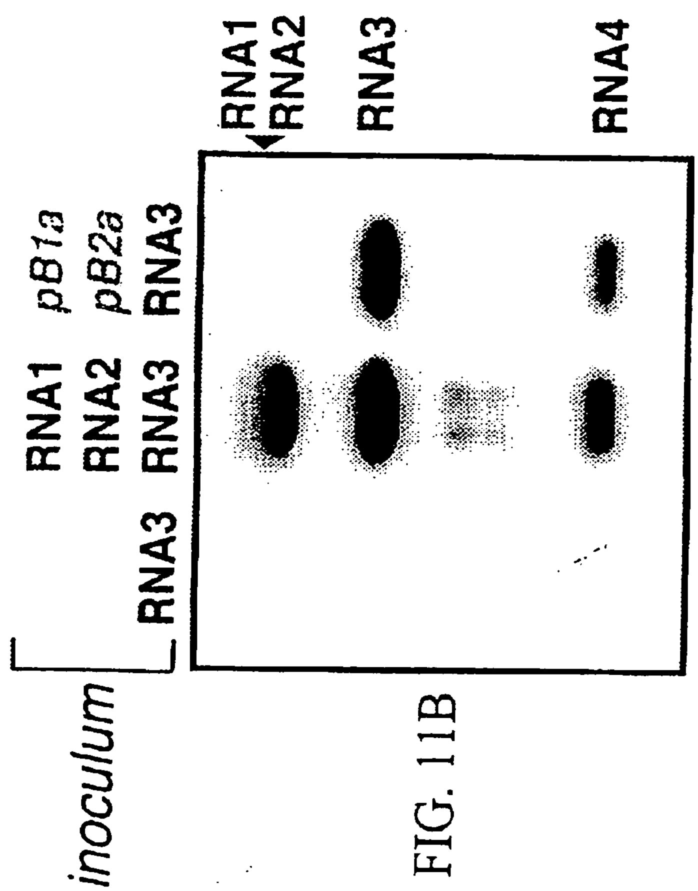

FIG. 11B

inoculum:

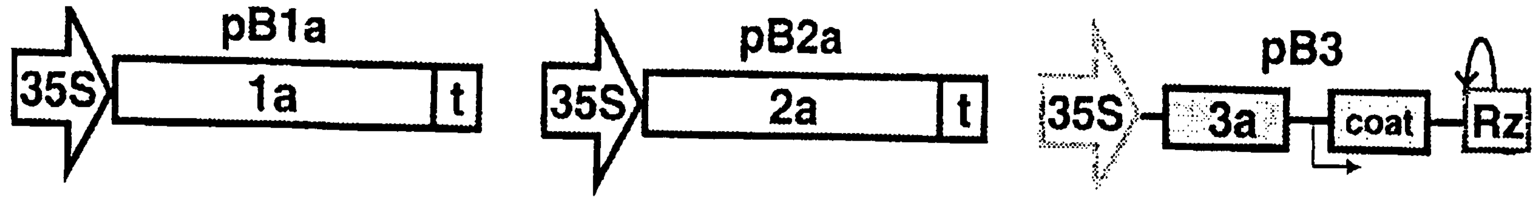

FIG. 12A

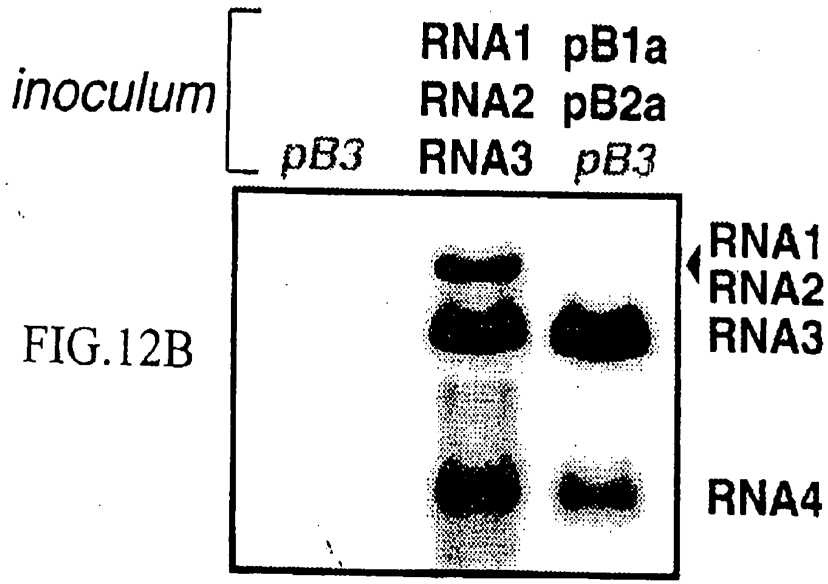

FIG.12B

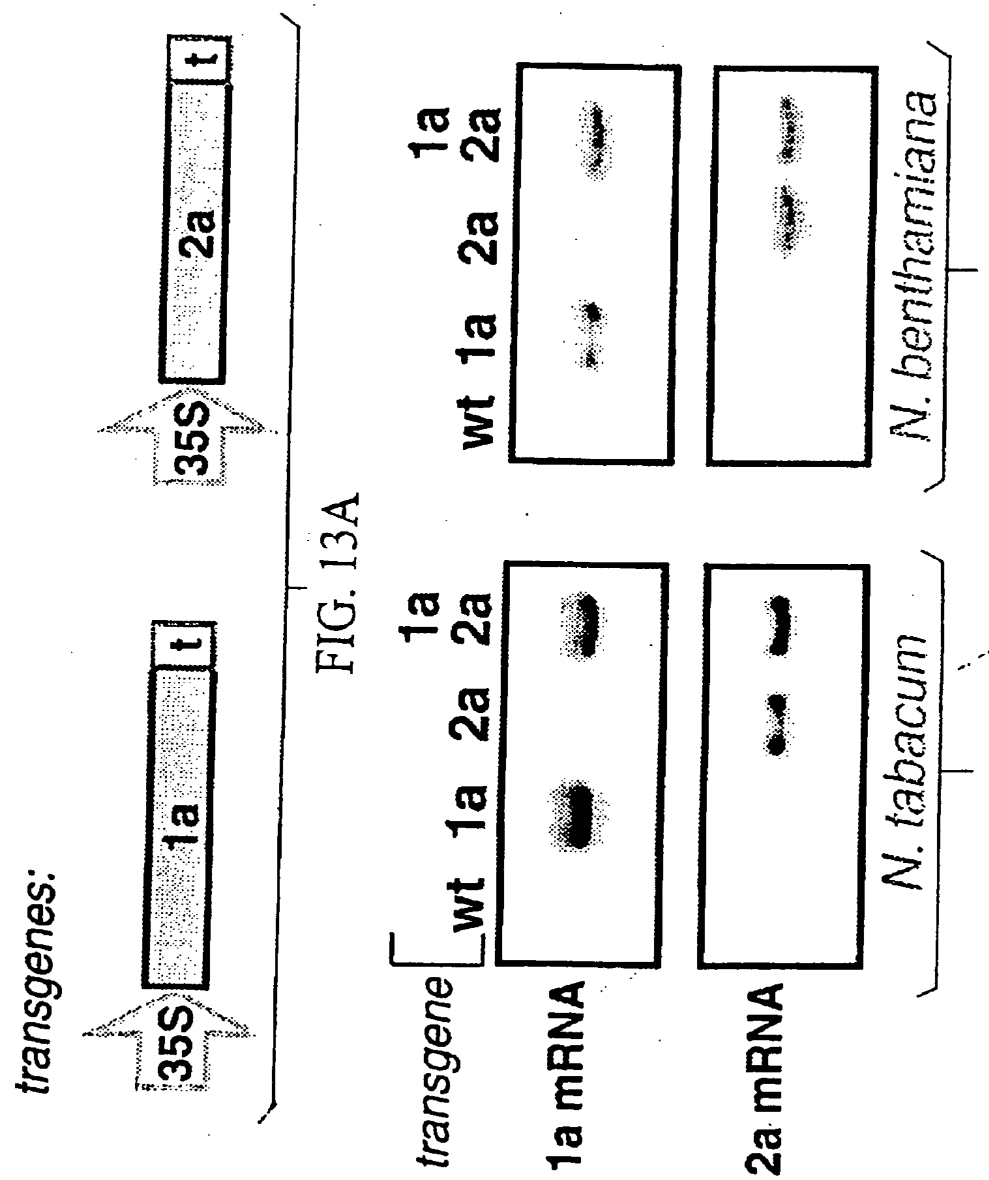

FIG. 13A

FIG. 13B

FIG. 13C

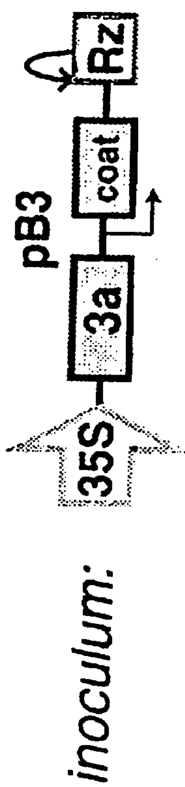

FIG. 14A

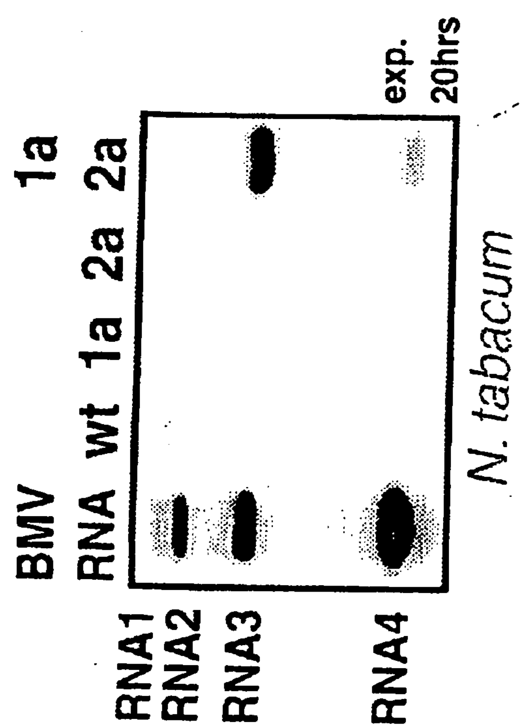

FIG. 14B

FIG. 14C

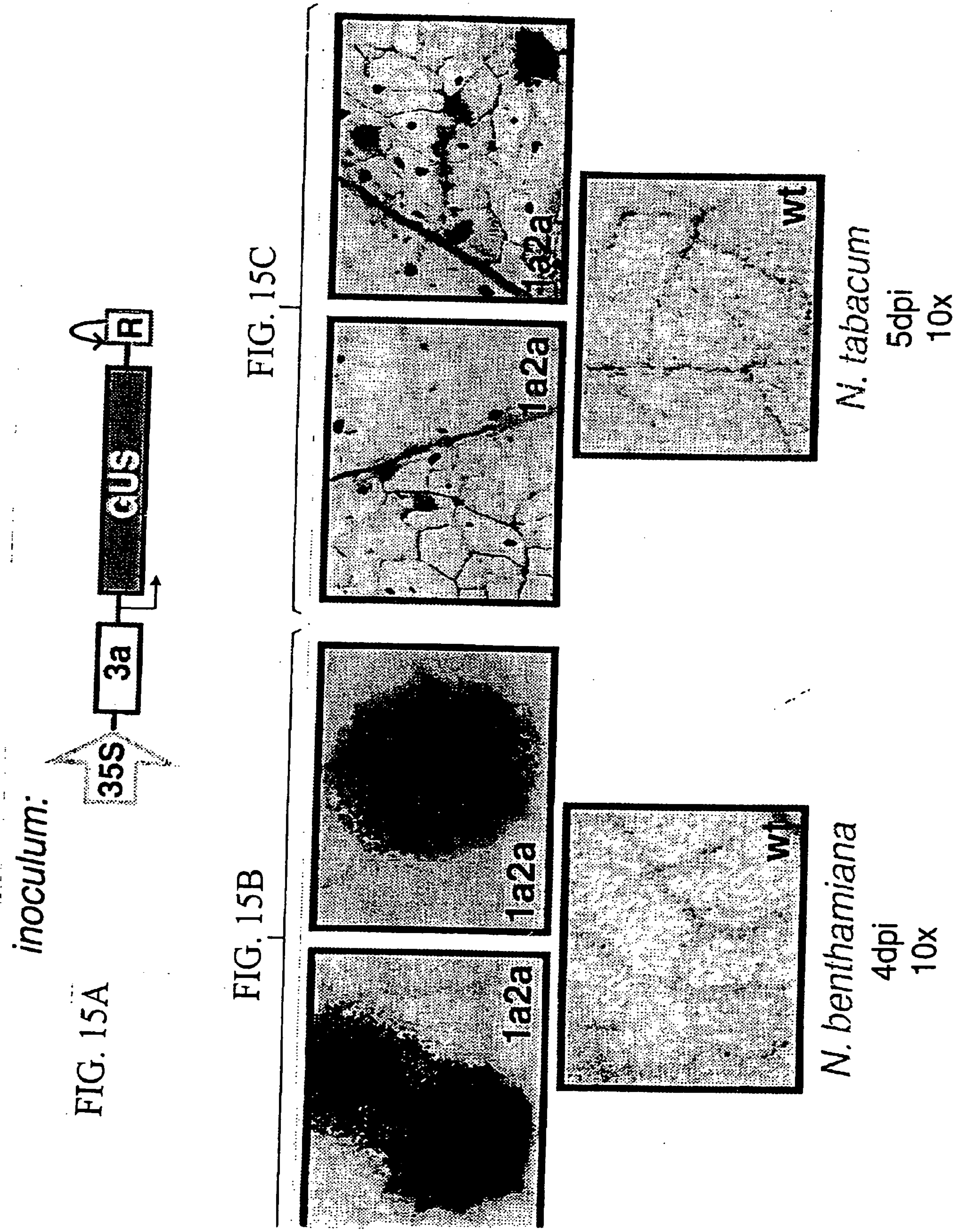

FIG. 15A

FIG. 15B

FIG. 15C

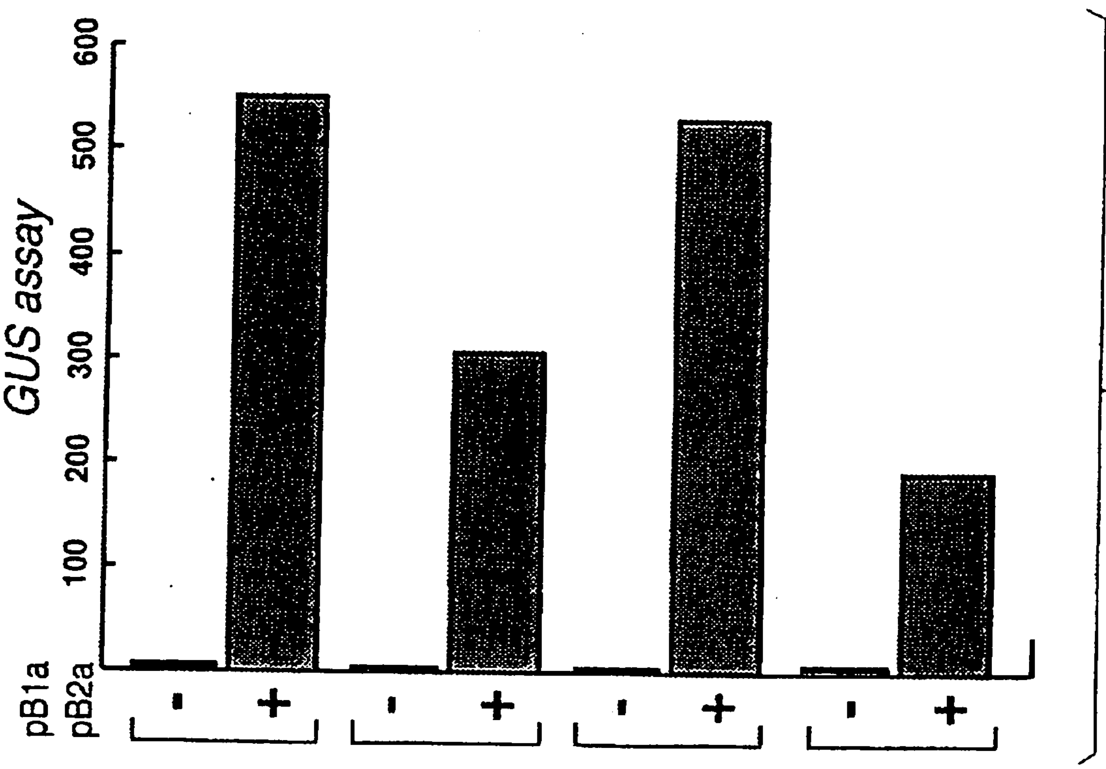

FIG. 16B

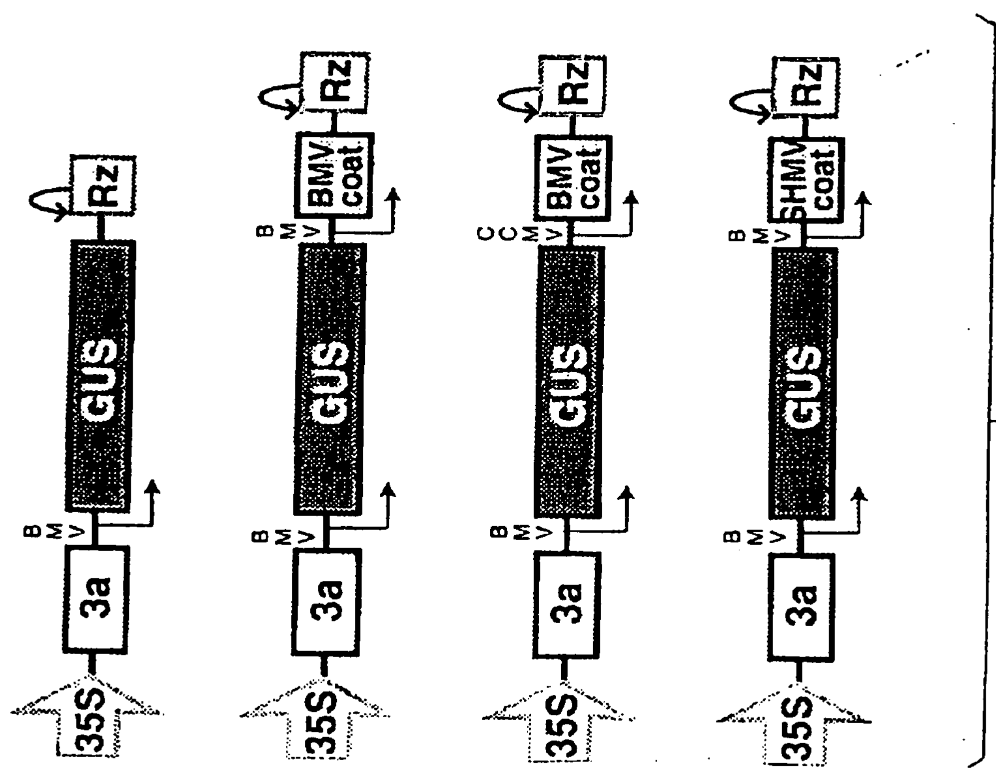

FIG. 16A

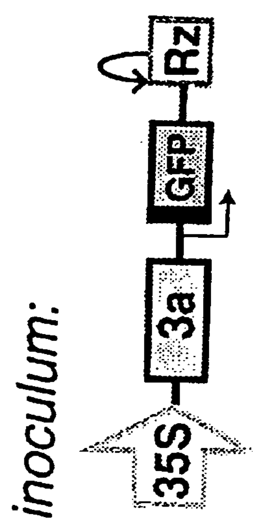

FIG. 17A

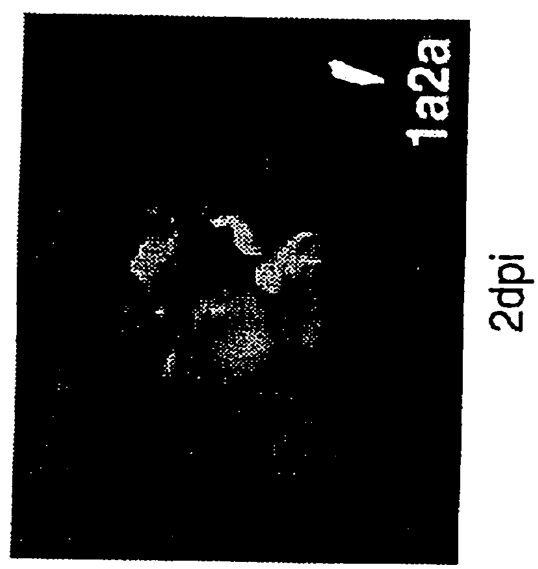

FIG. 17B

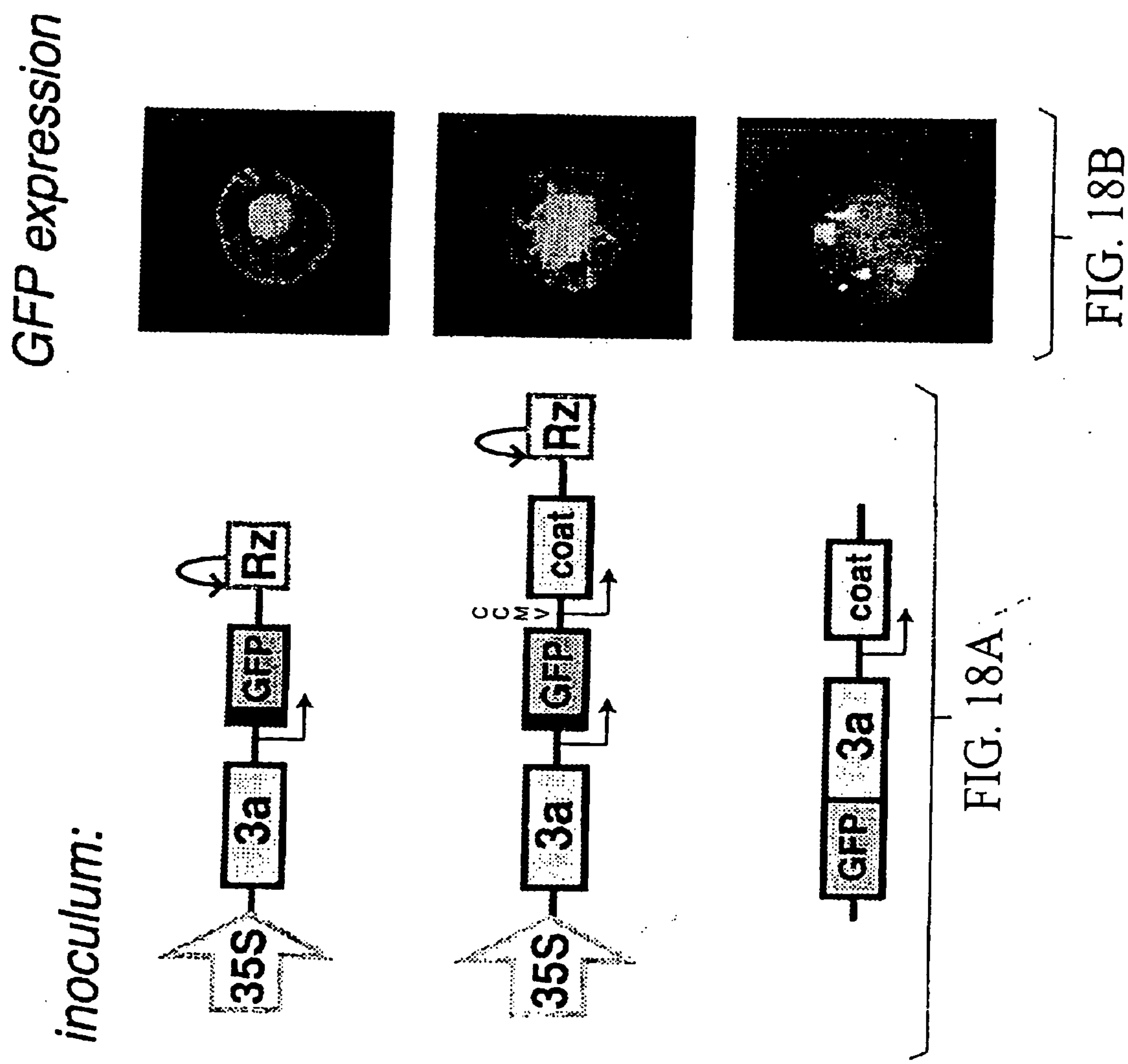

FIG. 18B

FIG. 18A

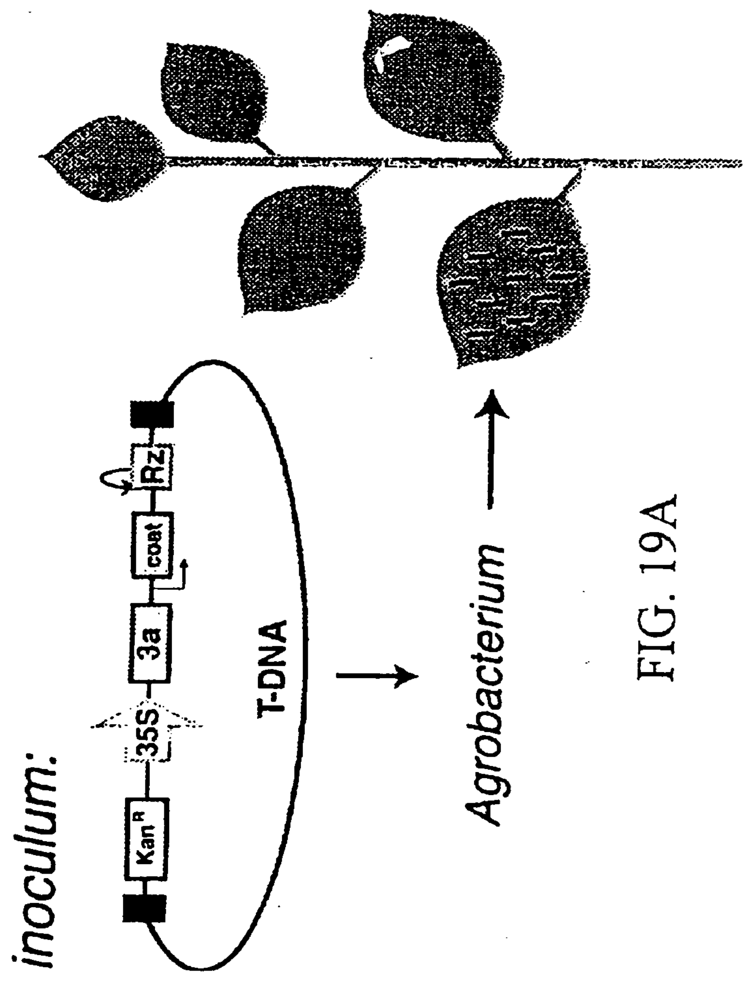

FIG. 19A

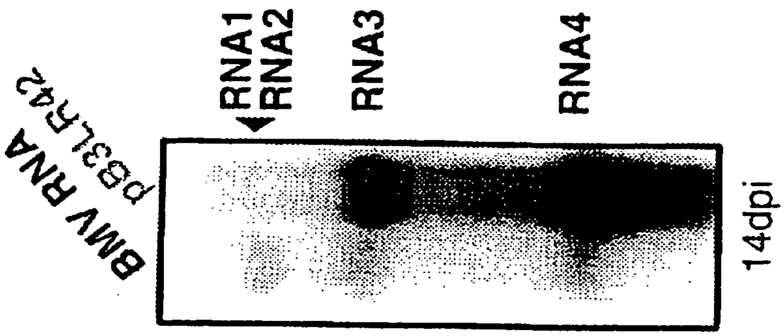

FIG. 19B

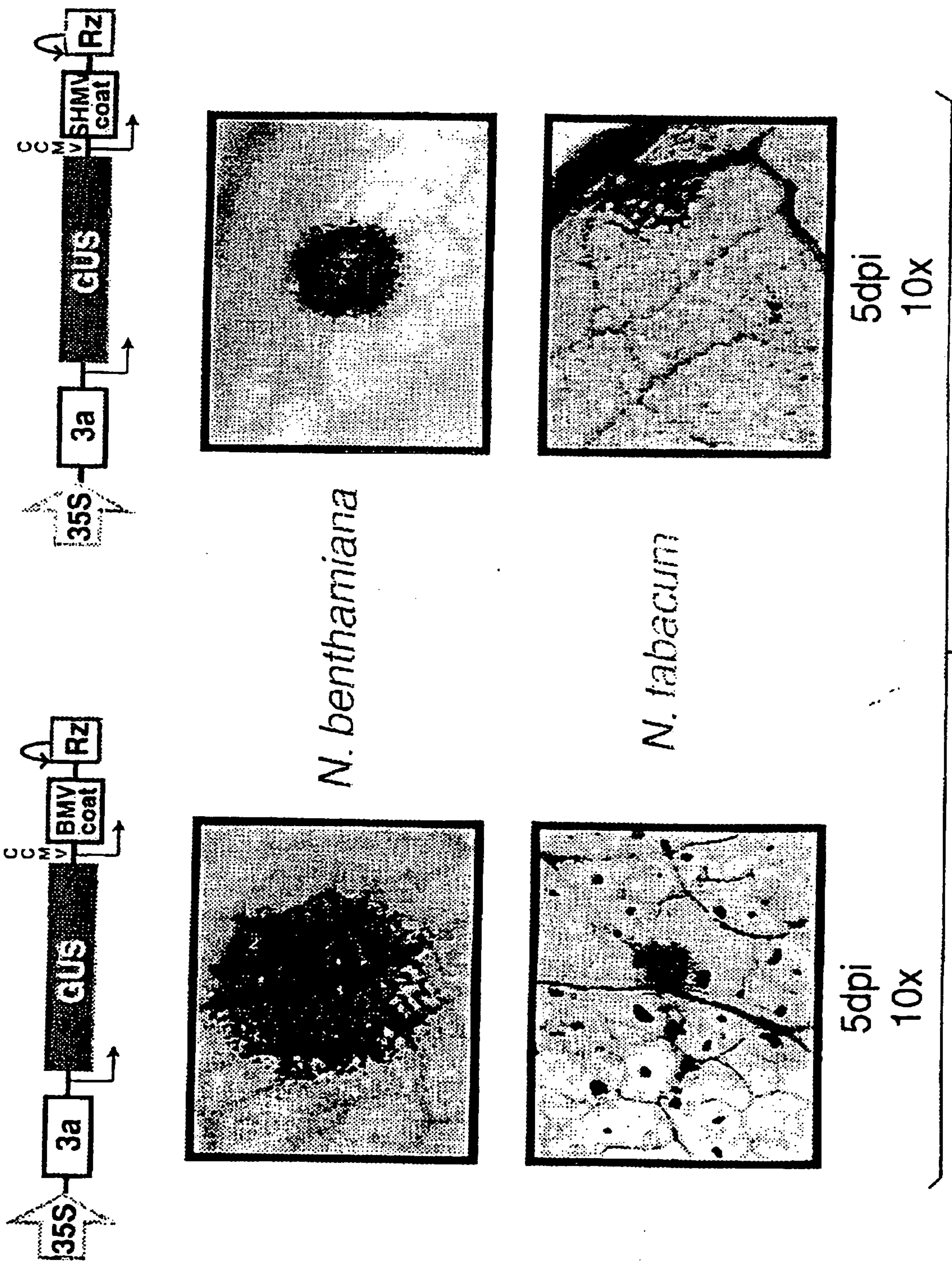
35S
3a
GUS
CCMV
BMV coat
Rz
35S
3a
GUS
CCMV
SHMV coat
Rz
N. benthamiana
N. tabacum
5dpi
10x
5dpi
10x

FIG. 20

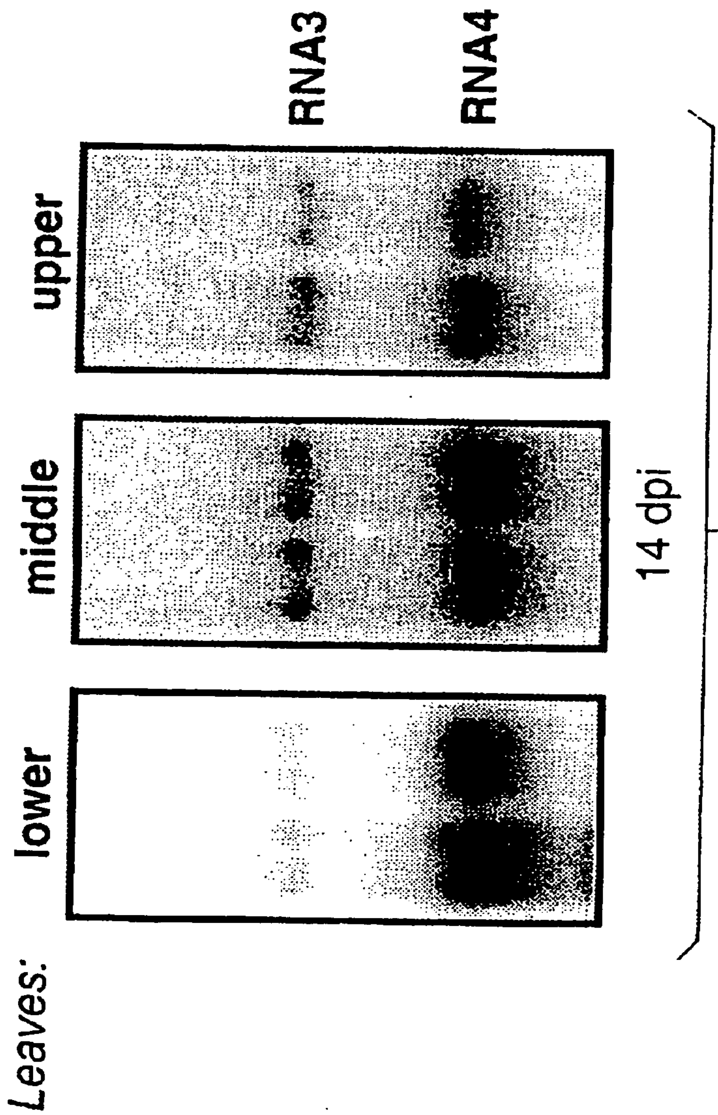

FIG. 21

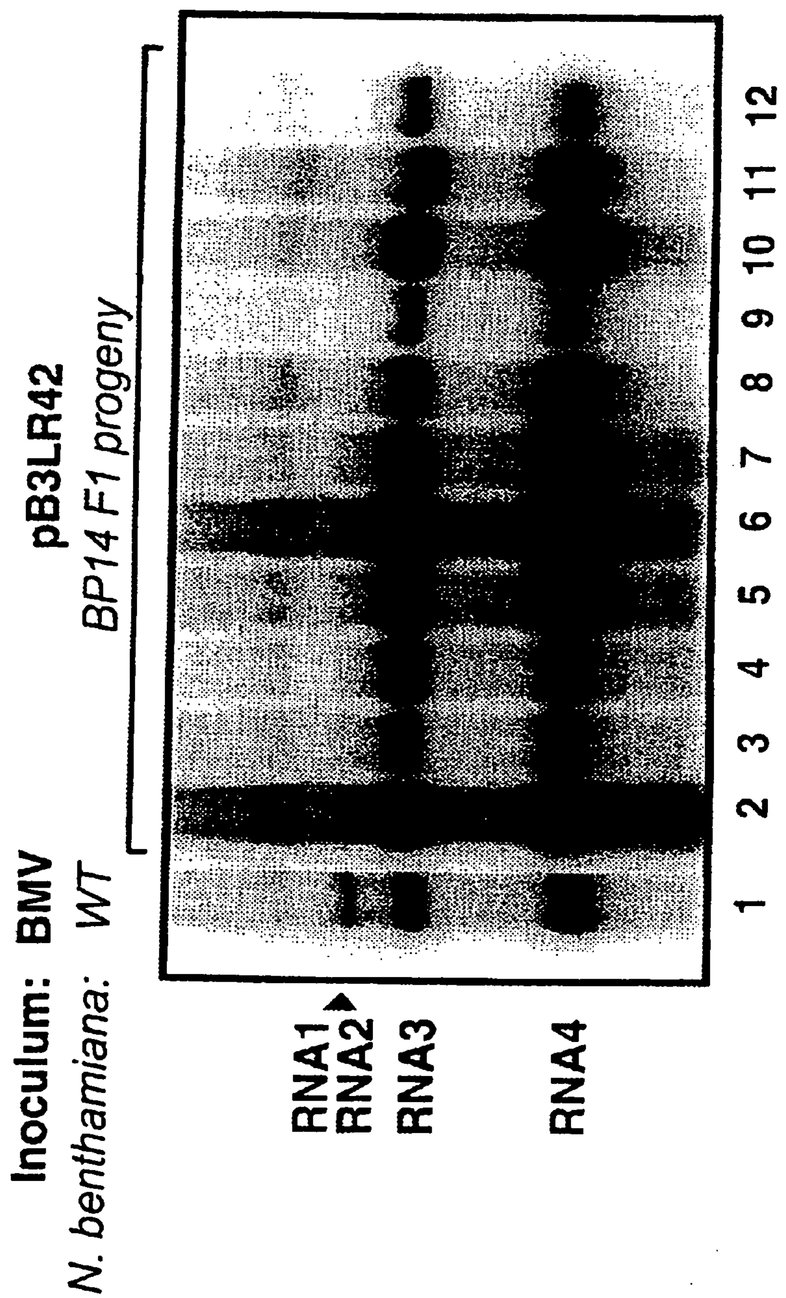
Inoculum: BMV
pB3LR42
N. benthamiana: WT
BP14 F1 progeny
1 2 3 4 5 6 7 8 9 10 11 12
RNA1
RNA2
RNA3
RNA4

FIG. 22